EFFECTIVE NUCLEAR DELIVERY OF THERAPEUTIC MOLECULES

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Many strides have been made towards utilizing gene therapy to treat genetic disorders in humans. Low gene expression and formulation stability during storage have hindered the use of nonviral carriers thus far. The purpose of this research was to develop a cationic PLGA-*b*-bPEI micelle-based delivery system to co-deliver genes and small therapeutic drugs to the cell nucleus that could also be lyophilized for long-term storage and reconstituted as needed by simply adding water. Several PLGA-*b*-bPEI copolymers were synthesized and characterized for their potential to form reconstitutable micelle-based gene therapeutic delivery systems that showed enhanced nuclear delivery over other current cationic nonviral delivery systems.

The first section of this dissertation addresses the characterization of a reconstitutable charged polymeric micelle system to assess its suitability for gene therapeutics delivery. A PLGA-*b*-bPEI block copolymer was synthesized and characterized for buffering capacity, particle size, zeta potential, complexation ability, cytotoxicity, transfection efficiency and reporter gene expression, and retention of physicochemical and biological characteristics upon lyophilization and reconstitution. Results showed that micelles and micelle/pDNA complexes retained their physicochemical characteristics following lyophilization and reconstitution. Transfection levels were enhanced using reconstituted complexes compared to their fresh

counterparts without significant cellular toxicity even with pDNA doses up to 20μg, and transfection increased linearly with increasing pDNA dose.

The second section of this dissertation investigates the use of dexamethasone-loaded micelles for enhanced nuclear accumulation of a reporter gene. PLGA-*b*-bPEI polymers synthesized in the first section were used to prepare dexamethasone-loaded micelles and complexed with pDNA. Gene expression was significantly enhanced using dexamethasone-loaded micelle/pDNA complexes compared to blank micelle/pDNA complexes, particularly for complexation weight ratios less than 1. Dex-micelles achieved gene expression equivalent to the blank system using a fraction of the polymer (weight ratio 0.25 versus 1), indicating that dexamethasone increased nuclear accumulation of polyplexes. Gene expression was nearly equivalent to a bPEI control carrier, leading to future plans of co-loading the micelle system with other types of drugs and examining this system's suitability for specific applications such as treating patients suffering from asthma or other inflammatory diseases where dexamethasone treatment could be additionally beneficial for its anti-inflammatory properties.

This thesis is dedicated to my family,

for giving me the strength to persevere despite unbelievable odds.

“Permanence, perseverance and persistence in spite of all obstacles, discouragement, and impossibilities: It is this, that in all things distinguishes the strong soul from the weak.”

Thomas Carlyle, 1795-1881

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ADA-SCID	adenosine-deaminase severe combined immunodeficiency
ANOVA	analysis of variance
BCA	biocinchoninic acid
bPEI	branched polyethylenimine
CD	Cyclodextrin
DCC	1,3-dicyclohexyl carbodiimide
Dex	Dexamethasone
DI	deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FBS	fetal bovine serum
HBG	HEPES buffer containing glucose
HEPES	$_{D}$-glucose, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HSV-1	herpes simplex virus type 1
Luc	Luciferase
MMLV	Moloney murine leukemia virus
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MW	molecular weight
MWCO	molecular weight cut off
N/P	nitrogen to phosphate ratio
NHS	N-hydroxysuccinimide
NPC	nuclear pore complex
NTF	nuclear transport factor
PAGA	poly[a-(4-aminobutyl)-L-glycolic acid]
PAMAM	Polyamidoamine
PBS	phosphate buffered saline
PDMAEMA	poly(2-dimethylaminoethyl methacrylate)
pDNA	plasmid DNA
PEG	polyethylene glycol

PEI	Polyethylenimine
pHPMA	poly(N-2-hydroxypropyl methacrylamide)
PLGA	poly(lactide-co-glycolide)
PLL	poly(L-lysine)
Polyplex	polymeric gene complexes
RLU	relative luminescence unit
RNA	ribonucleic acid
RPMI	RPMI1640 medium
RT	room temperature
SD	standard deviation
TBE	Tris(hydroxymethyl) amine methane-boric acid-EDTA buffer
TEA	Triethylamine
WR	weight ratio

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CHAPTER 1

INTRODUCTION

1.1. Gene Therapy

Gene therapy refers to a potential therapeutic strategy that can be employed to treat diseases resulting from missing or mutated genes. This approach involves replacing the gene of interest with a normal copy, supplementing gene expression, or altering gene expression in an effort to rescue abnormalities caused by the presence of a defective gene [1-3]. The concept of gene therapy was first established over 50 years ago as a potential way to treat patients suffering from genetic defects by replacing defective DNA with exogenous “good” DNA [4]. Beginning in the 1960s, researchers began investigating ways to permanently introduce foreign DNA into mammalian cells after being inspired by work done in the 1940s where pneumococci had been transformed [5-12], and the first publications referring to gene therapy appeared in the early 1970s [13-15]. Policy makers began discussing the issues relating to and the ramifications of gene therapy in the seventies [4, 16-18], and in 1986 the National Institutes of Health adopted guidelines pertaining to human somatic-cell gene therapy protocols [19], paving the way for the first human clinical trials. Many important discoveries made in the 1980s including recombinant DNA techniques [20-23], the

development of retroviral vectors [24-34], and successful correction of a genetic disease phenotype *in vitro* [35-37] established human gene therapy as a conceptual reality. While attempts to treat patients with gene therapy occurred as early as 1970 [4, 38], it was not until 1988 that the first protocol for a human clinical trial for gene therapy received approval [19] and the first clinical study was performed in 1990 [39]. Early gene therapy models focused on the treatment of diseases caused by single genes such as coagulation disorders due to deficiencies in clotting factors [40-44], ADA deficiency [45-47], and many others but advancement in gene therapy techniques have made it possible to expand gene treatment further. To date, more than 900 clinical trials have been approved worldwide [48] and gene therapy has been investigated as a method to treat many multifaceted disease models including Parkinson's Disease [49], inflammatory arthritis [50], cardiovascular disease [51], muscle diseases [52], diabetes [53], and multiple types of cancer [54-57].

1.2. Gene Vectors

The basis of gene therapy involves the delivery of a normal gene or nucleic acid molecule into the cell or tissue affected by disease. This is a multidimensional problem characterized by several challenges that need to be overcome in order to achieve successful gene therapy. A fundamental component of this process is the delivery vehicle or gene vector used to carry and introduce the gene into the system. Initial attempts at gene transfer in the early 1960s were highly inefficient, making it evident that this was an important milestone that needed to be addressed in order to make

further advances. Towards the end of that decade, advancements were made towards understanding how viruses were able to transfer their viral genome, and this combined with the discovery of recombinant DNA motivated the focus of the next several years of research. By the early 1980s, several researchers demonstrated the ability to achieve 100% infection efficiency using retroviruses, and several studies demonstrated *in vitro* success in rescuing disease phenotypes in cells from human patients [35-37]. Subsequent studies employed several different cell types including epithelial cells, fibroblasts, and hepatocytes, optimizing and demonstrating the efficiency and efficacy of viral gene delivery.

1.2.1. Viral Vectors

Viral vectors can be derived from RNA or DNA viral vectors and include gamma-retrovirus (including Moloney murine leukemia virus), adenovirus, adeno-associated virus, lentivirus (including human immunodeficiency virus), herpes simplex virus, and others [58]. Because viral vectors are derived from viruses which have evolved natural mechanisms to deliver their genomes into cells, they display very high efficiency at transferring genes into cells (known as transduction) [3].

Retroviral vectors were the first virus-derived vectors to be used for gene delivery because they could integrate into the host genome with very high efficiency and produce stable gene transfer in the target cells which could potentially lead to long-term gene expression [59]. Retroviral vectors were among the first vectors used in human gene therapy trials starting in 1990 [60]. Among retroviral-derived vectors, the

most common is the Moloney murine leukemia virus (MMLV), a simple retrovirus that was one of the first retroviruses discovered and it remains widely used in gene therapy applications even today [58]. To date over 600 gene therapy clinical trials have used retroviruses as vectors to treat diseases ranging from adenosine-deaminase severe combined immunodeficiency (ADA-SCID) [61] to familial hypercholesterolemia [62] and even for developing tumor vaccines [63]. Some limitations of retroviral vectors are that integration can only occur in cells undergoing cell division [59] and they have a small gene cargo capacity (approximately 7.5 kb) [64]. Additionally many safety concerns have arisen after using retrovirus vectors; for example random integration into the host genome can produce harmful side effects such as activation of prooncogenes or in the cause of MMLV, the development of leukemia-like symptoms [59, 64, 65].

Adenovirus was the next viral vector to be utilized in humans, starting with gene therapy trials in cystic fibrosis patients in 1993 [66]. Adenoviruses have a higher packing capacity than retroviruses so they can be used to deliver larger genes [59]. They can enter a variety of cell types and produce gene expression in both dividing and non-diving cell types, thus overcoming some of the limitations associated with retroviral vectors. Adenoviruses produce very high gene transfer and unlike retroviruses they do not integrate into the host genome, making them useful for instances where high gene expression is desired for a short period of time. Adenoviruses enter the cell through receptor-mediated endocytosis [67] and can be amplified into very high titers in the laboratory [59]. Some clinical applications of adenovirus include the treatment of cystic

fibrosis [66], mesothelioma [68], and colon cancer [69]. The safety of adenovirus has also come under scrutiny because adenovirus can revert to its wildtype form, can leak viral proteins, and is highly immunogenic, so repeat applications to the host are problematic [64].

Adeno-associated virus are nonpathogenic, can infect a variety of cell types including quiescent cells, and like retrovirus it can integrate into the host genome [70]. It requires a helper virus for infection [71] and unlike retrovirus it integrates into a specific site on the human chromosome [72]. Adeno-associated viruses have been used to treat cystic fibrosis [73, 74], hemophilia [75], and Parkinson's Disease [76]. Some disadvantages of this vector are the difficulty in producing large amounts of vector and its very limited gene carrying capacity (<5 kb) [77]. The original strains of adeno-associated virus have been modified using recombinant techniques to expand upon some of the shortcomings associated with the original virus but there are still some safety concerns associated with these vectors.

Lentivirus is a complex virus system considered to be a subset of retrovirus. Lentiviral-vectors can be used to transfect both dividing and nondividing cells, and have several advantages over adenoviruses and adeno-associated viruses. Lentiviral vectors have larger coding capacity [78], similar transduction efficiency as adenovirus and adeno-associated virus at lower titer levels [79], and reduced immunogenicity *in vivo* compared to other viral-derived vectors [80, 81]. Lentiviral vectors produce stable gene expression in quiescent cells such as neurons [82] and hematopoetic stem cells [83], and

have been used to treat many neurological diseases including Parkinson's Disease [76] and Huntington's Disease [84], control tumor growth [85], and even prevent HIV infection [86]. The most commonly used lentivirus is derived from human immunodeficiency virus (HIV).

One of the most promising viral-derived vectors discovered in the quest for an ideal gene therapy vehicle is the herpes simplex virus, derived from herpes simplex virus type 1 (HSV-1). It is of great interest because of its size and the complexity of its genome [58], allowing the delivery of larger transgenes than nearly any other viral-vector [87]. Vectors derived from HSV-1 have several favorable features including the lack of integration into the host genome, complexity of the virus genome, the capacity to infect the nervous system including an ability to spread from neuron to neuron and establish latent infections in neurons, and their ability to be controlled using anti-herpetic agents such as acyclovir or ganciclovir [87, 88]. HSV-1 has been used to develop vaccines [89], anticancer agents/treatment [90], and for the treatment of chronic pain [91].

Although viral vectors have comprised the majority of successful gene therapy efforts in humans, success has come with a price. As mentioned above, numerous concerns have arisen regarding the safety of viral vectors. Although they are extremely efficient, the very features that make viral based gene delivery systems attractive also limit their usage because of unwanted side effects (Table 1.1). Both retroviruses and lentiviruses have the ability to integrate themselves into the host genome, which is

Table1.1. Summary of Viral-based Gene Delivery Vectors

Viral Vector	Advantages	Disadvantages
Adenovirus	• large host spectrum (mitotic/post-mitotic cells) • high titers • high transduction efficiency • episomal viral genome • wildtype causes mild disease • non-enveloped • large coding capacity	• immunogenic • reversal to wild type • transient gene expression in dividing cells (clearance of the episome) • leakage of viral proteins • toxicity
Retrovirus	• high transduction rates *in vitro* • large host spectrum among dividing cells • system is well studied and known • genomic integration /long term gene expression • vector proteins are not expressed in the host • relatively large coding capacity	• immunogenic • requires dividing cells • risk of insertional mutagenesis • risk of reversion to the wild type • potential inactivation by complement fractions in the serum • low titers • low delivery rates *in vivo* • *ex-vivo* application necessary • virus recombination
Adeno-associated virus (AAV)	• *in vivo* and *ex vivo* administration possible • genomic integration/long term gene expression • large host spectrum (mitotic & post-mitotic cells) • no associated human disease • preferential site-directed integration of DNA	• low coding capacity • limited DNA packaging capacity • integration is not always site-directed • immunogenic

Table1.1. (continued)

Viral Vector	Advantages	Disadvantages
Herpes Simplex virus (HSV)	• episomal • may produce latent infection during whole life span • accommodates large inserts • high titers	• immunogenic • different viruses have different selectivity • EBV is oncogenic • latent virus activation • low transduction efficiency • transient expression by available vectors • developing system
Lentivirus	• high transduction efficiency in vitro • *in vivo* administration possible • large coding capacity • infects and transduces mitotic and post-mitotic cells • long duration of gene expression • large coding capacity	• system is poorly understood • low efficiency in vivo • insertion mutagenesis • virus recombination • potential inactivation • *ex vivo* administration necessary for keratinocytes

beneficial for long-term gene expression but causes problems when integration occurs at nondiscriminate sites, and in the case of MMLV can cause leukemia-like symptoms [59, 64, 65]. Furthermore, this nonspecificity can give rise to other potentially harmful effects in the event that random integration results in mutagenesis or oncogensis. Whereas retroviruses integrate themselves into rapidly dividing cells, adenoviruses can only integrate themselves into nondividing cells, and therefore need to be readministered since their presence is not propagated by cell division. However, readministration of adenoviral gene vectors elicits an immune response that can be deadly [92]. Although viral-based carriers have been used in approximately 70% of the human clinical trials to date [48], alternative nonviral based vectors are rapidly gaining popularity in an effort to produce a therapeutic strategy that minimizes negative side effects. Over the last two decades, research has turned towards the development of alternative strategies of gene delivery vehicles in an effort to preserve the high efficiency of viral-based systems while minimizing dangerous side effects and concerns of the viral delivery vectors as questions regarding their safety in patients have emerged.

1.2.2. Nonviral Vectors

Avoiding carrier integration into the host genome, immunogenicity, and tumorigenicity are among the issues that are at the forefront of research aimed towards creating nonviral based carrier systems. In addition, viral-based systems are limited by the size of DNA they can carry [64, 77], are not easy to manufacture reproducibly [93],

and are more difficult to customize for targeting purposes [94]. Nonviral vectors are not derived from viral sources and thus may overcome many of the concerns associated with viral-based systems. There are numerous benefits for using nonviral delivery systems including nonimmunogenicity, nontumorgenicity, biocompatibility, the ability to target the carrier to particular cells/tissues by attaching ligands to the carrier surface, and others. In addition to these benefits, there is no restriction in the size of the DNA that can be delivered, which was a major negative associated with the use of viral-based vectors. Nonviral vectors can be classified into two major categories: lipid-based or polymer-based systems [95].

1.2.2.1. Naked DNA-based Systems

The simplest method of nonviral delivery is the direct transfer of genes using plasmid DNA [96]. This method of gene transfer has been most successful when the DNA is injected directly into the tissue of interest, particularly in muscle tissue. Injection of naked plasmid DNA has been used to successfully express leptin and erythropoietin in muscle tissue [97], and even vascular endothelial growth factor to promote blood vessel development [98]. Because gene expression is generally low with this method, it has also been used to develop vaccines. Generating antiviral immune responses to infectious agents has led to the development of DNA vaccines against infectious diseases such as AIDS [99] and tumor vaccines that induce antitumor immunity by directly inhibiting tumor growth factor or eliciting an immune response [100]. However, gene expression is quite low using this method, likely because DNA is not protected

from degradation, and tissue diffusion is quite poor, limiting its application for gene therapy merely to delivery via direct intratissue injection.

1.2.2.2. Lipid-Based Gene Carriers

Lipid-based carriers are typically composed of neutral or charged lipids and can assume different organized structures including liposomes (Figure 1.1), emulsions, micelles, and others [97]. The earliest carriers were based on neutral or anionic liposomes which are generally nontoxic and fairly compatible with biological fluids, making them a good candidate for systemic delivery [97]. Neutral and anionic liposomes have been widely used for drug delivery applications [101] and early studies were able to establish the feasibility of using liposomes as a gene delivery vehicle [102]. Unfortunately, these liposomes were not successful as gene delivery carriers [97]. Since DNA is also negatively charged, encapsulation efficiency was very low [103]. In addition, these carriers lacked endosomal escape mechanisms and had insufficient interactions with cells, leading to low transfection. Because of technical difficulties associated with increasing DNA encapsulation within the vesicle, focus shifted towards cationic liposomes [97].

Cationic liposomes have several advantages over neutral/anionic liposomes. Because of they are composed of lipids with a positively charged head group (Figure 1.2) they are able to complex with DNA through charge interactions instead of having to encapsulate the DNA within the vesicle as with neutral/anionic lipsomes [97].

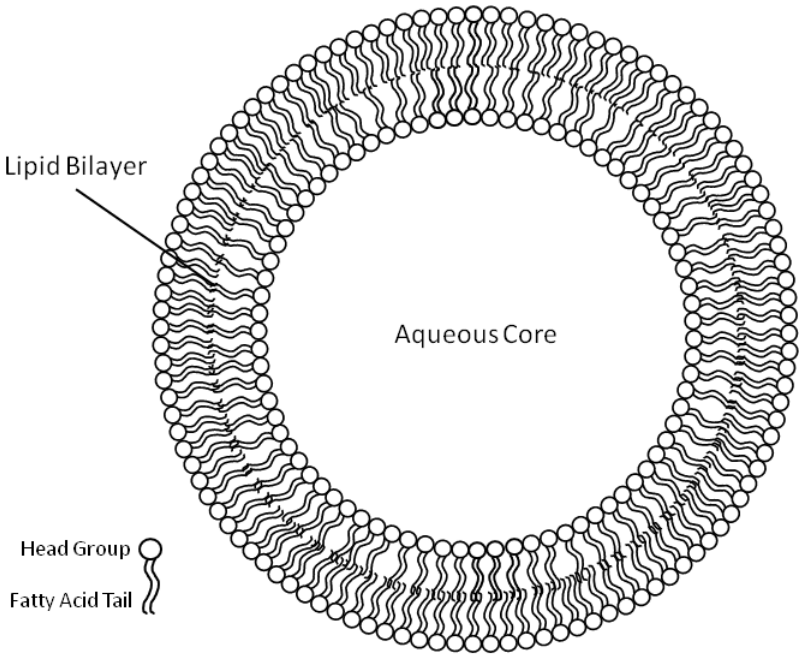

Figure 1.1: Schematic representation of a liposome

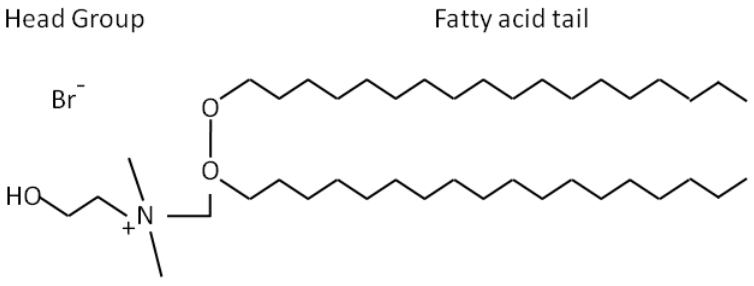

Figure 1.2: Representative cationic lipid structure

During preparation, complexes can be formed such that the surface charge remains slightly positive, increasing interactions between the positively-charged complex and the negatively-charged cell membrane [97]. In addition, the composition of lipids can be selected such that the complex has a built-in endosomal escape mechanism which would increase DNA release into the cytosol after endocytosis [104]. Lastly, complexation between DNA and the cationic lipids may protect DNA from enzymatic degradation and physical damage [105]. Cationic liposomes can form different types of liposome/DNA complexes including spherical structures, rod-like structures, spherical aggregates, and compact nonaggregating particles [97]. However, the structure of liposome/DNA complexes can change over time and is affected by several factors including charge ratio between lipid and DNA, concentration, and solution pH. Cationic liposome/pDNA complexes can be administered using several different methods including intratumor injection [106], airway administration [107, 108], intraperitoneal administration [109], and intravenous administration [110]. Cationic liposomes have proven to be somewhat successful as gene carriers, and have been involved in gene therapy clinical trials for several types of cancer [111-117] and cystic fibrosis [118-121]. But transfer of functional DNA molecules into cells by DNA-loaded liposomes or virosomes is not very efficient. The difficulties involved with the intravenous use of cationic liposome-DNA complexes arise as a result of their underlying transfection mechanism. Cationic liposomes form complexes with the negatively charged DNA via charge interactions. Optimal transfection of the liposome-DNA complex relies on the

presence of excess positive charge, which is required for efficient interaction with the negatively charged cell membrane. Neutralization of excess positive charge in the liposome-DNA complex by negatively charged serum proteins may result in decreased transfection efficiency. Also, DNA might be released from liposome-DNA complexes by anionic molecules in the serum, rendering DNA more susceptible to enzymatic degradation. Furthermore, serum proteins can induce an aggregation of liposome-DNA complexes, leading to rapid clearance of liposome-DNA complexes from the blood by the reticuloendothelial system (RES). These problems, together with others, severely limit the intravenous application of cationic liposome-DNA complexes.

1.2.2.3. Polymer-Based Gene Carriers

Polymer-based systems have many appealing biological characteristics such as excellent biocompatibility, low immunogenicity, and no tumorigenicity that make them well-suited as a gene-carrier material. Other important characteristics include the ability to functionalize and customize polymers by adding features such as cell-targeting moieties, endosome-disrupting agents, and degradable architectures, allowing the flexibility to adapt polymer-based systems to suit any situation.

The first reported use of polymer/DNA complexes for gene delivery by Wu *et al.* in 1988 where they administered polylysine/DNA complexes systemically and were able to detect gene expression in the liver [122]. Since that time many different polymers have been investigated including polyesters [123-127], poly-L-lysine (PLL) [128-133], poly(N-2-hydroxypropyl methacrylamide) (pHPMA) [134-137], and polyethyleneimine

(PEI) [138-141]. Polymer/DNA complexes (polyplexes) can assume various structures including toroidal structures [142, 143], spherical globules [144], small well-defined particles [145], nano- and microparticles [146, 147], and DNA-loaded polymeric microspheres [148]. The structure can vary depending upon the length and size of DNA, ionic strength of the solvent, and the concentration of the polymer and DNA during mixing. One method to avoid aggregation is to create cationic polymers with hydrophobic (hydrophilic) segments [149, 150]. Cationic polymers generally condense DNA more effectively than neutral polymers due to electrostatic interactions between the positively-charged polymer and negatively-charged DNA. Unlike liposomes where lipid selection is limited, the variety of polymers available is limitless as novel polymers can be designed to adapt to any situation with features including endosomal-disruption mechanisms [151], targeting moieties to increase cellular uptake, degradation mechanisms to decrease cytotoxicity, and others. Polymer vectors can be constructed either from degradable or nondegradable polymers and this selection varies according to the desired application of the vector.

Polymeric micelle-based carrier systems and more specifically cationic-micelle systems have gained interest in recent years due to their unique core-shell architecture where hydrophobic segments segregate into an inner core surrounded by the hydrophilic shell or corona (Figure 1.3), and narrow size distribution. These charged micelles have high colloidal stability due to steric repulsion of their corona (shell) and often assume a spherical shape [152]. The capacity to load drugs within the micelle core

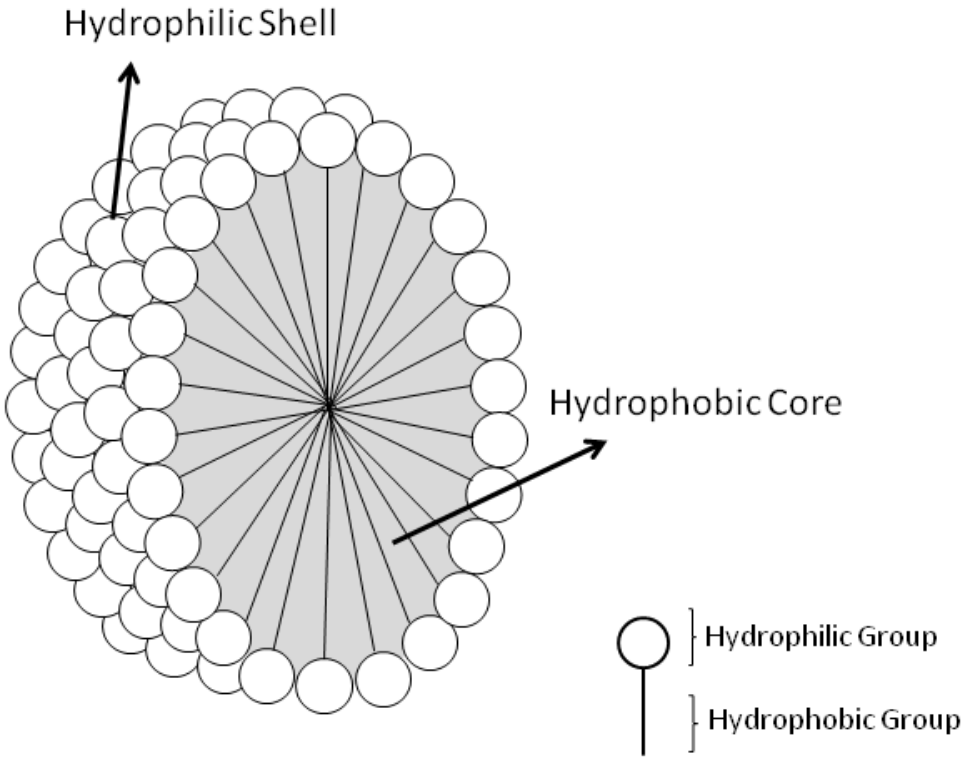

Figure 1.3: Schematic representation of a micelle

and the ability to modify the biological and physicochemical properties of the carrier by functionalizing the shell makes cationic micelles particularly attractive vehicles for gene delivery applications. The hydrophobic core has been used to load hydrophobic drugs such as paclitaxel whose use had been limited due to poor solubility in water; using micelle-based carrier systems shielded the drug from an aqueous environment and allowed higher local accumulation of the drug in the tissues of interest. Numerous studies have been conducted where the corona of the micelle was modified by binding ligands or other agents to increase targeted delivery of the carrier [153, 154], or creating polymers with stimuli-sensitive properties that can reduce side effects and increase therapeutic efficacy [154, 155].

A major limitation in current nonviral vectors including polymer-based carriers is the low levels of cell transfection and gene expression compared to what viral-based vectors can achieve. Many combinations of existing and novel polymer systems have been investigated in an effort to increase cell transfection through a variety of mechanisms including increasing cellular uptake through targeting and prolonged systemic circulation, engineering endosomal escape mechanisms, and enhancing nuclear penetration. The most commonly studied cationic polymer systems include poly-L-lysine (PLL), poly(2-dimethylaminoethyl methacrylate) (PDMAEMA), polyamidoamine (PAMAM), and poly(ethylenimine) (PEI). These polymers have been well characterized in terms of their gene condensation and gene binding abilities and

transfection properties *in vitro* and *in vivo* [149, 156-161]. Numerous other types of cationic polymer systems have been investigated including various types of polyesters [123-126], polyurethanes [162, 163], and phosphor-containing polymers such as poly(phosphazenes) [164-166].

1.2.2.4. Poly-L-Lysine (PLL)

Poly-L-lysine (PLL) was the first cationic polymer to be studied for gene delivery (Figure 1.4). A targeted system directed towards hepatocytes was created by complexing PLL with asialoglycoprotein to form polyplexes. Since hepatocytes have a receptor for galactose-terminal asialo(glycoproteins), these complexes could be taken up into the cell via receptor-mediated endocytosis [167]. This system was also tested *in vivo* and showed preferential gene expression in the liver but no activity in other organs [122]. Another group conjugated PLL to transferrin which entered cells through

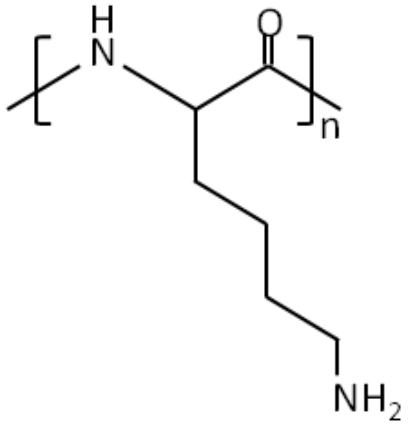

Figure 1.4: Chemical structure of poly(L-Lysine) (PLL)

receptor-mediated endocytosis and resulted in high levels of gene expression in human leukemic cells [168, 169]. Specific gene expression was observed in mouse lung endothelial cells using a PLL-antibody-DNA complex system which also targeted mouse lung tissue *in vivo* [170]. Although polylysine by itself is a very poor gene-delivery vehicle, conjugation with targeting ligands greatly increased its delivery efficiency both *in vitro* and *in vivo*. Since those initial studies, PLL has been used to transfect very diverse cell populations including tumor cells [171], pituitary cells [172], thyroid follicular cells [173], human blood monocyte-derived macrophages [174], and many others. Many studies regarding polyplex formation and endolysosomal escape were based on polylysine-DNA complexes. Due to its low transfection efficiency, and high toxicity, polylysine is generally used as a basis for comparison of more promising polymers. Some newer polymers derived from PLL are showing some promise as gene carriers including poly[a-(4-aminobutyl)-L-glycolic acid] (PAGA), a biodegradable polymer designed by Kim's group [125, 175].

1.2.2.5. Poly(amidoamine) (PAMAM)

A second generation of polymeric gene carriers was developed based on poly(amidoamine) (PAMAM) dendrimers (Figure 1.5). PAMAM polymers have a large number of secondary and tertiary amines, allowing the polymer to act as a proton sponge [176]. PAMAM dendrimers form highly condensed complexes with DNA, and generally generation 6 or 7 dendrimers have been used for gene transfer [177]. Dendrimers can be activated or partially degraded prior to complexation and this

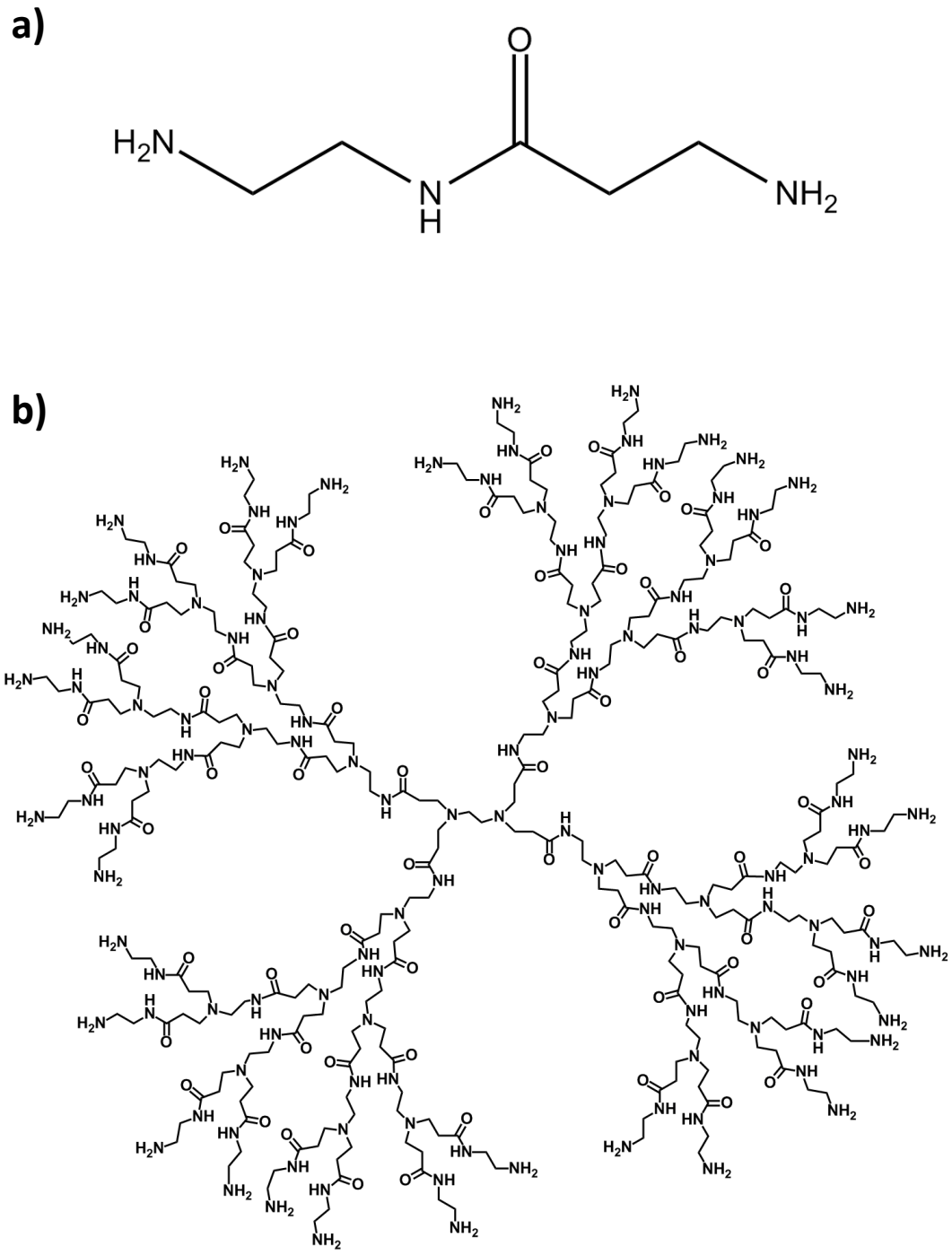

Figure 1.5: Chemical structure of (a) amidoamine and (b) PAMAM dendrimer

process has increased transfection efficiencies over nonactivated dendrimer systems [178]. Activated PAMAM dendrimers have been used to create two commercial transfection reagents by QIAGEN – SuperFect® Transfection Reagent and PolyFect® Transfection Reagent [177]. Both of these reagents have been used in laboratory gene transfer experiments to successfully transfect numerous cell types including primary aortic smooth muscle cells [179], neuronal cell lines [180], and fibroblast and epithelial cells [181]. Because of their generally good biocompatibility, nonimmunogenicity, and fairly high gene delivery efficiency [182-184], PAMAM dendrimers have been used in several gene therapy studies *in vivo* [185-187].

1.2.2.6. Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA)

Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) is another well-studied cationic polymer that has shown promise as a gene carrier system (Figure 1.6). It has relatively low cytotoxicity, and its amine groups confer high buffering capacity to the polymer. The amine groups also allow the polymer to effectively bind and condense DNA to form discrete particles [188-192]. PDMAEMA can be conjugated to other polymers to create carriers which can serve dual purposes such as oligonucleotide delivery and bioimaging [193], or for combinatorial delivery by co-loading micelle carriers with both oligonucleotides and small chemical drugs [194]. This polymer has also been used to create temperature and pH-sensitive hydrogels for drug release in response to stimuli [195].

1.2.2.7. Cyclodextrins

Cyclodextrins (CDs) are oligosaccharide molecules comprised of 6-8 glucose units linked together to form a ring with a hydrophilic exterior and a hydrophobic interior (Figure 1.7). There are several hydroxyl groups on the ring which can be used to manipulate the structure for different applications [196]. They have been used extensively in the pharmaceutical industry to improve the physical properties of drugs (stability, solubility, bioavailability) [197]. Two additional features also contribute to increased drug bioavailability; their membrane absorption enhancing properties and their ability to stabilize biomolecules by shielding them from nonspecific interactions in physiological media [198-200]. CDs have also been shown to increase oligonucleotide stability against endonucleases and can modulate undesirable side effects such as immune stimulation [201, 202]. The 7-glucose cyclodextrin in particular, termed β–cyclodextrin (Figure 1.7), has been shown to increase gene expression in rat lung, which is attributed to enhanced membrane permeation capabilities in this tissue. In addition, these formulations showed no apparent toxicity *in vivo* [203].

1.2.2.8. Polyethylenimine (PEI)

To date, PEI remains as the gold standard for polymer-based carriers because it is able to achieve *in vitro* gene transfection rates comparable to those of viral vectors [204]. The high density of amino groups on the polymer gives it a high density of positive charge, making it very effective at condensing DNA and giving it a high affinity

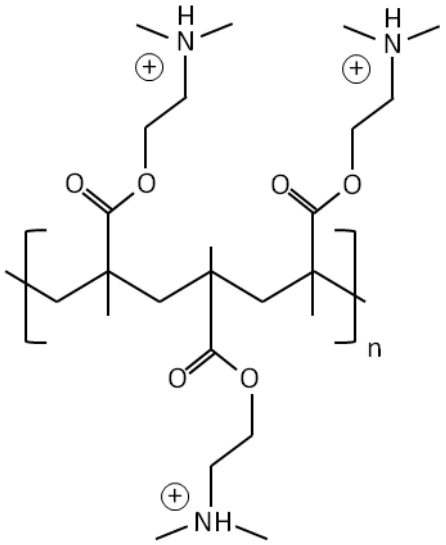

Figure 1.6: Chemical structure of poly(2-(dimethylamino)ethyl methacrylate)

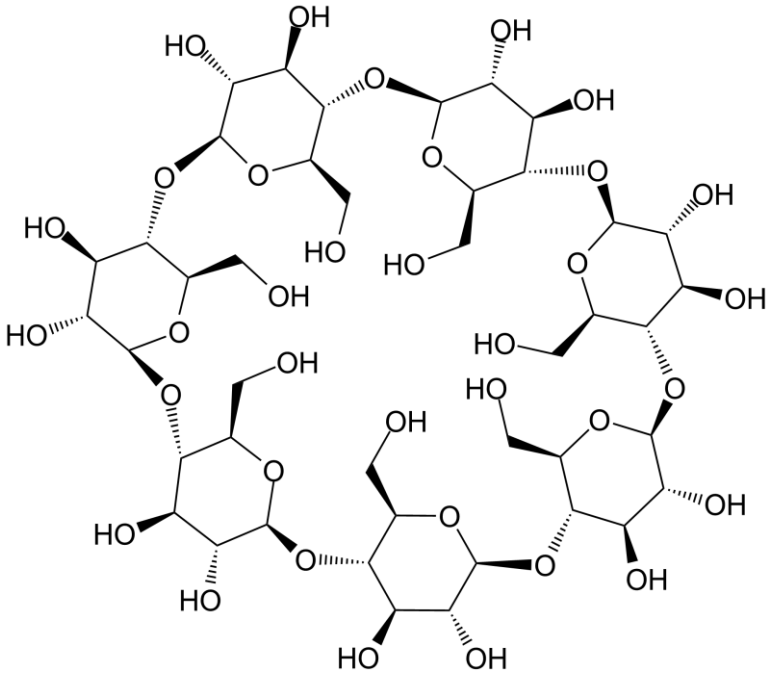

Figure 1.7: Chemical structure of β–cyclodextrin

to interact with negatively charged cells. The amino groups also act like “proton sponges” and endow the polymer with high buffering capacity inside the endosome [205, 206]. This leads to increased osmotic pressure and ion flux which in turn causes swelling and finally leads to endosome rupture. Membrane disruption can also occur through interactions between the positively charged PEI particles and the negatively charged endosomal membrane [160, 206, 207]. It has been shown that PEI can condense DNA to form small particles, and protects DNA from shearing or endosomal degradation to deliver intact DNA into cells [208]. PEI can be linear or branched and both forms are widely used because they provide high levels of gene expression (Figure 1.8) [209]. PEI-based DNA particles have been used to treat bladder cancer in humans with encouraging results [210] and were able to produce permanent gene expression in the respiratory tract without undesirable expression in other tissues [211].

One unfortunate drawback of highly positively charged polymers including PEI is they are quite cytotoxic, due to aggregation and reaction with erythrocytes and blood components *in vivo* [212, 213]. Because PEI is very effective in mediating gene delivery, several groups have focused their efforts towards altering PEI to increase its biocompatibility while maintaining its high tranfection rates. Direct modifications to PEI include acetylation by Forrest *et al.* to convert primary and secondary amines into secondary and tertiary amides, which increased gene delivery activity considerably over unmodified PEI [214].

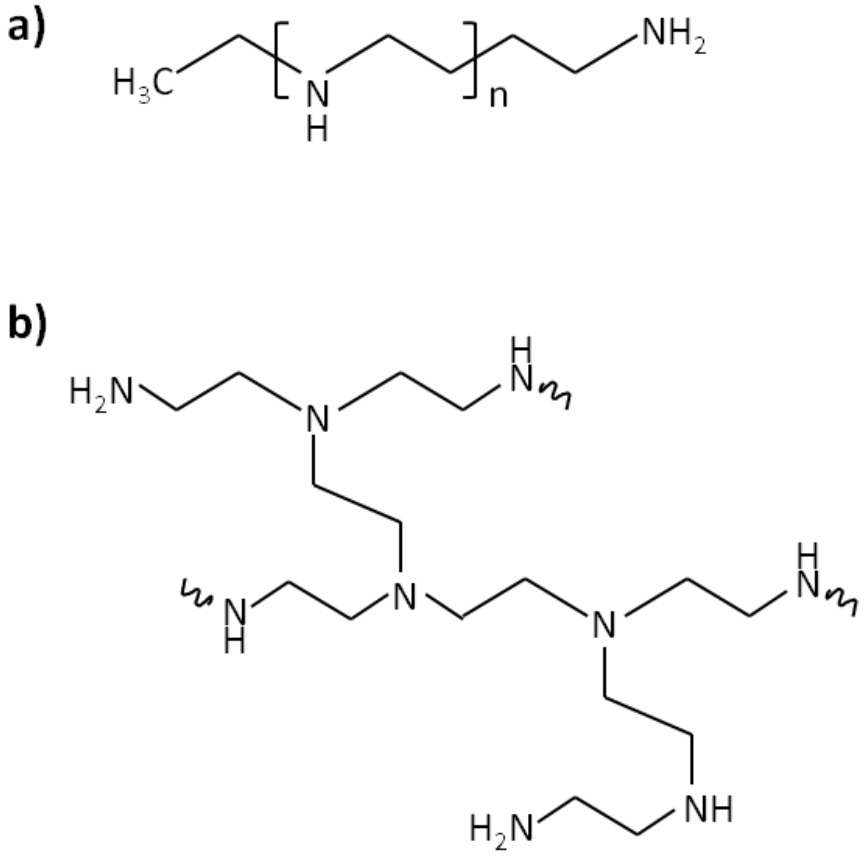

Figure 1.8: Structure of (a) linear and (b) branched polyethylenimine (PEI)

Layer-by-layer techniques emerged to better protect DNA from premature degradation after finding that low transfection rates correlated to exposed DNA in the complexes. While progress has been made in identifying favorable characteristics that enhance DNA packaging in the carrier, cellular uptake of the carriers, and DNA release once inside the cell, low levels of transfection and expression compared to what viral-based carriers can achieve remains as a major limitation. One reason for lower efficiency is the number of extracellular and intracellular barriers encountered by the complex that need to be overcome to achieve successful transfection. Viral-based vectors are already equipped to deal with these barriers because the original virus had evolved coping mechanisms; however nonviral based carriers need to be designed to survive the barriers encountered.

1.2.2.9. Polymeric Micelle-Based Systems

Micelles, particularly polymeric micelles, have gained popularity in recent years as a carrier system for delivering many different agents. Micelles have been used as carriers for diagnostic agents, improving aqueous solubility of hydrophobic drugs, and site-specific drug delivery. An attractive feature is the ability to customize the system by selecting polymers with particular features such as sensitivity to various stimuli like pH or light. Using amphiphilic molecules, molecules that contain both hydrophilic groups and hydrophobic groups, allows the self-assembly of micelles upon exposure to solvent. Amphiphilic copolymers can be formed by conjugating a hydrophilic or polar polymer to a hydrophobic or nonpolar molecule. Upon solvent exposure, these amphiphilic

copolymers spontaneously form micelles with a core-shell architecture (Figure 1.3). In an aqueous environment the hydrophobic blocks will aggregate together forming a core compartment away from the solvent, and the hydrophilic blocks will align towards the solvent and form a shell. This segregated compartment structure allows various poorly water-soluble molecules or drugs to be loaded into the core, increasing their solubility and bioavailability in the body. Advantages of using micelle-based systems include ease of preparation, increased circulation time, decreased toxicity, increased drug solubility, targetability, and enhanced penetration of tissue. The use of amphiphilic polymers in particular also minimizes micelle disintegration upon dilution and can potentially prolong drug delivery [215, 216]. Polymer options for the hydrophobic block include aspartic acid, propylene oxide, caprolactone, and L-lysine among others. Hydrophilic polymers include polyethyleneoxide, polyethyleneglycol, polyvinylalcohol, and polyethylenimine and others [217]. To date, micelle systems have been used to solubilize a myriad of poorly water-soluble drugs including diazepam [218], indomethacin [219], adriamicin [220-222], anthracycline antiobiotics [223], polynucleotides [224, 225], and doxorubicin [226].

Polymeric micelles have several benefits over other delivery systems such as microparticles and liposomes. Microparticles usually are micron-sized in diameter, so they are not suitable for many applications due to limited uptake in cells. In addition, they are not as effective at solubilizing poorly soluble drugs compared to micelle systems. Polymeric nanoparticles tend to aggregate easily, so although the actual

particle size may be less than 100nm, aggregation causes larger particles. They also have significant burst release due to the increased surface area in the formulation, and drug loading can be limited in these small particles.

Micelle-based systems are more versatile than systems using polycation polymers alone. Micelle characteristics can easily be adapted to better suit a particular application by changing either block in a copolymer. For example, one can increase the capacity of the hydrophobic core by changing the hydrophobic block and thus better solubilize poorly soluble drugs [227-229]. Certain polymers contain charged groups that can cause endosomal disruption, leading to better delivery intracellularly [228].

Polymeric micelles have also been used as gene delivery vehicles in an effort to develop more effective nonviral based delivery systems. If the hydrophilic block of a polymer is a cationic polymer such as polyethylenimine then the micelle can effectively condense negatively charged plasmid DNA (pDNA) into micelle/pDNA complexes (polyplexes). This protects the pDNA from degradation by enzymes or through hydrolysis until delivery. In addition, there is the option to modify the micelles with targeting ligands to increase site-specific accumulation or delivery. Using polycations alone to condense pDNA can result in uncontrollable biodistribution in the body because of their charge [230], increased cytotoxicity, and the polyplexes may also contain free polycations which can cause instability problems [206]. However, when pDNA is complexed with cationic micelles made from amphiphilic polymers, the resulting polyplexes are quite small (50-200nm) [152], soluble in aqueous solution, and

the repulsive nature of the corona prevents the aggregation of micelle particles, giving these systems high colloidal stability [152, 231]. During micelle preparation, any remaining free polymer is removed during the dialysis process, and the resulting micelles usually have lower cytotoxicity compared to their constituent polymers alone [231, 232]. DNA complexed with polymeric micelles to form polyplexes are protected from degradation by nucleases [231] and have reduced interaction with blood components during circulation [152]. An important benefit of using micelles as gene carriers is that gene expression can be higher and more sustained than is achieved with other delivery vehicles, which is especially true when micelles are equipped with targeting moieties which allow preferential accumulation at a site of interest [233, 234].

1.3. Delivery Barriers

Several obstacles must be overcome before successful transfection can occur. The first hurdles encountered are extracellular, during the delivery of the carrier to the host. Depending upon the route of administration, extracellular barriers can include polyplex stability during systemic circulation in the blood *in vivo* (or in cell culture medium for *in vitro*) and accumulation at nontarget sites or clearance by other mechanisms. For *in vivo* applications, there is the potential for the polyplex to become coated with serum proteins or DNA degradation from proteases in the blood which could decrease the efficacy of the polyplex. For *in vitro* applications polyplexes need to remain stable in the culture medium which contains serum proteins that can cause dissociation of the polyplex. Thus gene packaging and serum stability of the polyplexes

are very important. Cell-specific targeting can greatly enhance polyplex accumulation in the tissue or cells of interest.

Once polyplexes are successfully internalized into the cell, they encounter intracellular barriers including escape from the endosomal compartment before being degraded, transport through the cytoplasm, and finally localization into the nucleus. Depending on the size of the polyplex, cell entry can occur by endocytosis and polyplexes are contained within endocytic vesicles known as endosomes. These endosomes can either shuttle their cargo back to the cell surface to be expelled via exocytosis or acidify and become lysosomes where polyplexes can be partially or completely degraded. Only DNA that is able to escape from the endosomes into the cytoplasm can be delivered to the nucleus. Endosomal escape can be mediated by conjugating pH-sensitive synthetic or fusogenic peptides to the polymer to disrupt the endosomal membrane, treating cells with buffering agents such as chloroquine which works for *in vitro* applications but would be unfeasible for *in vivo* applications, or by utilizing polymers known to have endosomolytic activity such as PAMAM and PEI.

If endosomal escape is successful, polyplexes are released into the cytoplasm and must navigate their way to the nucleus. The concentration of proteins, cytosolic nucleases, other organelles, and the microtubule network within the cytoplasm present yet another barrier to efficient gene delivery. Current mechanisms of polyplex transport through the cytoplasm need to be properly characterized in order to properly design and improve polymeric vectors to overcome this obstacle. The final barrier to gene

transfection is entry into the nucleus through the double-bilayer nuclear membrane. Depending on how the polymer is designed, DNA delivery could occur once the entire polyplex enters the nucleus and the DNA is released inside the nucleus, or the polyplex could decomplex in the cytosol following endosomal escape so only the DNA is translocated into the nucleus.

1.4. Nuclear Delivery

Entry into the nucleus of a cell is highly controlled and occurs through tightly regulated pores. Molecules enter and exit the nuclear compartment only through these pores decorating the surface of the nuclear membrane. These pores are filled with assemblies called nuclear pore complexes (NPC). NPCs are assemblies comprised of about 30 different proteins that assemble to form a molecular mass approximately 50 MDa in size [235]. The NPC is a cylindrical structure composed of eight spokes surrounding a central tube that connects the nucleoplasm to the cytoplasm [235]. The central channel has a minimum diameter of 35 nm and controls the entry and exit of molecules from the nuclear compartment. Molecules that are smaller than 35 nm in diameter (or less than 40 kDa) can freely diffuse through the nuclear pore complex; this includes small molecules such as water and oxygen, metabolites such as glucose, and ions [235-237]. Molecules larger than 35nm (or greater than 40 kDa) are unable to freely diffuse through the nuclear pore complex; instead they require a nuclear localization signal and the assistance of soluble nuclear transport factors to enter into the nucleus [235-237].

1.4.1. The Nuclear Pore Complex

The nuclear pore complex (NPC) is a 50 MDa assembly comprised of approximately 30 different unique proteins that spans the nuclear envelope to connect the cytoplasm to the nucleoplasm. The NPC contains octagonal radial symmetry, with a main central core structure that is anchored into the nuclear envelope by eight spokes [235, 238]. The spokes join to form three main rings that surround a central tube. An inner ring (or central ring) is located in the middle of the NPC and is sandwiched by two outer rings, one on nucleoplasmic side and another on the cytoplasmic side. These rings together form the central tube around 35 nm in diameter through which transport occurs [235]. Peripheral filaments are attached to the core structure, filling the central hole and emanating into the nucleoplasm and cytoplasm. These filaments come together to form a basket-like structure on the nuclear side of the NPC [238].

1.4.2. Nuclear Import

All molecular entry into and out of the nuclear compartment occurs through the nuclear pore complexes. Entry can occur via two mechanisms, passive diffusion or energy-mediated transport. Small molecules such as ions and metabolites are freely permeable through the nuclear pores, while larger macromolecules require the assistance of nuclear transport factors and transport signals in order to enter or exit the nucleus [235, 238]. Nuclear transport factors bind to nuclear localization signals found on the cargo and then translocate through the nuclear pore. Translocation occurs in a three-step process. First, nuclear transport factors (NTF) recognize and bind to the

nuclear transport substrates. These NTFs include importin α and β, and RanGTP among others. Next, the NTF-cargo complex docks to the NPC by binding to filamentous Phe-Gly nucleoporins and translocates through the NPC. Finally, after reaching its target compartment the NTF-cargo dissociates [235]. If molecules do not meet one of the aforementioned criteria, they are excluded from the nuclear compartment. This includes gene/polymer complexes, and is one of the reasons gene transfection using nonviral vehicles such as polymers remains low. An important factor for increasing transfection efficiency is to enhance delivery of polyplexes inside the nucleus of the cell. This can be accomplished by creating small polyplex particles which can easily diffuse through the nuclear pore, conjugating nuclear localization signals or nuclear proteins to the surface of the polyplex, or utilizing receptor-mediated transport by binding ligands to the polyplex such as glucocorticoids which cause translocation into the nucleus upon binding to the glucocorticoid receptor, thereby increasing accumulation in the nucleus and enhancing overall gene transfection.

1.4.3. Glucocorticoid Receptors

Glucocorticoid hormones are hydrophobic steroid hormones that are widely expressed in the cell [239, 240]. The binding of these molecules to the glucocorticoid receptor stimulate a number of profound physiologic effects many of which are necessary for life [241]. Every cell contains glucocorticoid receptors, and the binding of glucocorticoids to the receptor forms an activated ligand-receptor complex that translocates into the nucleus, and one result is the triggering of gene transcription and

expression [241]. In addition, it has been discovered that conjugating the particular glucocorticoid ligand dexamethasone to polymeric gene carriers such as polyethylenimine or polyamidoamine dendrimers caused an increase in gene expression that correlated to increased nuclear localization of the complexes [242]. Dexamethasone was even conjugated to DNA directly before transfecting cells and tested for its ability to increase gene expression. It was found that this Dex-DNA conjugate was increasingly localized in the nuclear compartment, and resulted in higher gene expression than when no dexamethasone was used. This indicates that dexamethasone directly increases the nuclear accumulation of DNA and could be beneficial for enhancing the nuclear accumulation of micelle/pDNA complexes.

Dexamethasone is a steroid hormone (Figure 1.9) that after binding to the glucocorticoid receptor causes the nuclear pore complex to dilate followed by translocation into the nucleus [241, 243]. It has been suggested that the binding of glucocorticoid ligands such as dexamethasone to the glucocorticoid receptor may aid the entry of polyplexes into the nucleus by ligand-receptor-mediated transport. The presence of dexamethasone has been shown to facilitate the transport of polymer/DNA complexes into nucleus, and conjugating dexamethasone to the polymer increased transfection efficiency [242, 244]. By adding this molecule to polyplexes either by conjugation to the polymer backbone or by loading into the complex, transport of polyplexes into the nucleus becomes enhanced. An added benefit of dexamethasone is

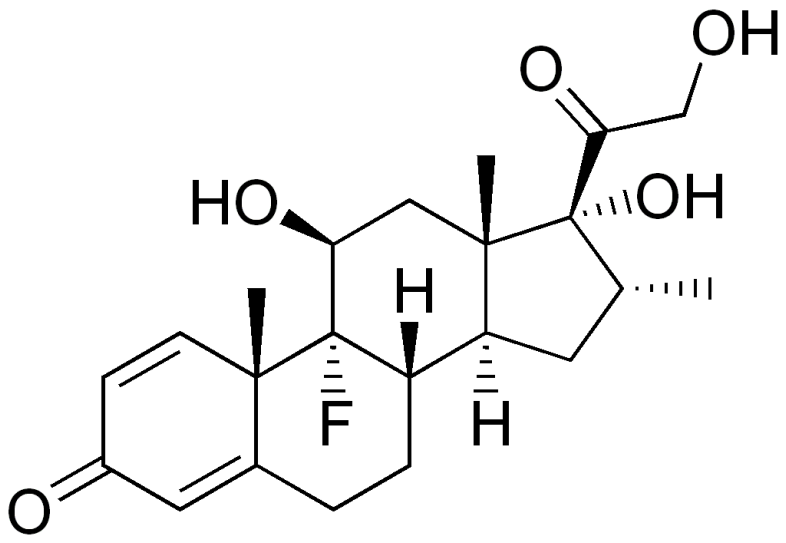

Figure 1.9: Dexamethasone

that it may also suppress an immune response in the host, which could help decrease potential side effects resulting from the treatment. Because dexamethasone is a small hydrophobic molecule, it can easily be loaded into the hydrophobic core of a micelle creating a drug-loaded micelle, which can then be complexed with DNA to create a dual-loaded carrier system.

1.5 Clinical Needs – Formulation Stability During Storage

From a therapeutic perspective, in addition to creating a gene delivery carrier that can transfect cells with high efficiency, the formulation must also be suitable for clinical applications. Important considerations include ease of administration, drug concentration, and stability of the formulation during long term storage. Many

formulations made from liposomes or polymer-based systems exist as colloidal liquids, which are generally stable for short periods of time [245]. However, some polymer systems are designed to be gradually degradable by hydrolysis so long-term storage in liquid form is not a viable option for these types of systems [246]. Additionally, many formulations can become unstable if kept in liquid form during long-term storage due to a variety of issues including degradation of the nonviral vector and/or drug [247], formation of insoluble aggregates [248], unwanted drug release [249], and loss of bioactivity [250]. One method to overcome the stability limitations of storing liquid formulations is to lyophilize the formulation, allowing storage in a powder formulation. When the formulation is required, it can be reconstituted though the simple addition of water or buffer. While some success has been seen using this method, many groups have additionally added cryoprotective agents [251, 252] or modified the formulation in some way such as through PEGylation [253, 254] or physical cross-linking [255]. Unfortunately these formulations underwent changes in their characteristics following reconstitution such as increases in particle size [251]. The ideal scenario would be to have a formulation that can be lyophilized without using any special additives, reconstituted by simply adding water or buffer, and most importantly retain all of its original chemical, physical and biological properties.

1.6 Rationale for the Study

Thus far, research has shown that gene therapy holds great promise as a therapeutic option for people suffering from diseases caused by mutated or absent genes. However, current gene vectors are derived from viruses which have raised many safety concerns due to harmful side effects such as immunogenicity and severe toxicity as seen with negative outcomes from several clinical trials. This has increased interest in nonviral carriers. Lipid-based carriers have been tested in several gene therapy trials but are plagued by limited DNA encapsulation and low transfection rates. Polymer-based gene carriers are a promising alternative delivery vehicle, because they avoid the negative issues associated with viral systems and polymeric micelle-based carriers using cationic polymers are particularly attractive because of their well characterized structure and drug loading capabilities. Long-term storage and utility of current gene-carrier formulations in the clinic is also of concern because many systems cannot be stored long-term in aqueous form and attempts to lyophilize various formulations has uncovered difficulties in formulation integrity during lyophilization and following reconstitution.

This study focuses on the creation of a reconstitutable cationic polymer micelle-based gene delivery system that could be used as a dual delivery system and targeted to the nucleus of the cell. Three different PLGA-bPEI copolymer systems were investigated and compared to a control delivery system based on branched polyethylenimine $bPEI_{25kDa}$ alone. Although transfection efficiencies achieved using the copolymer systems

were slightly lower than levels achieved using $bPEI_{25kDa}$ alone, the copolymer systems were considerably less toxic than $bPEI_{25kDa}$ alone. Three different copolymer systems were used to make blank micelles and dexamethasone-loaded micelles that were characterized in terms of physicochemical and biological properties.

Chapter 2 outlines the creation and evaluation of a reconstitutable charged polymeric micelle gene delivery system created from the copolymer $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$. The hypothesis was that this particular copolymer could form micelles and micelle/pDNA complexes that retained their physicochemical characteristics following lyophilization and reconstitution and these complexes could be used to successfully transfect cancer cells with an exogenous gene.

Chapter 3 outlines the creation and evaluation of three different dexamethasone-loaded polymeric micelle systems with a PLGA-*b*-bPEI structure and their ability to enhance nuclear accumulation and gene expression in MCF7 cells. The hypothesis was that co-loading dexamethasone and complexation with pDNA would create a dual-delivery system. Following cellular uptake and endosomal escape, dexamethasone would release from the micelle core and dilate the nuclear pores, allowing increased entry and accumulation of polyplexes into the nucleus. This would increase gene transfection and subsequent gene expression, paving the way for more successful gene therapeutics delivery.

Chapter 4 will summarize the scientific merits of the above studies and discuss some future directions for this research.

Finally, the appendix summarizes work completed on a prior dissertation project related to optimizing a bioartificial pancreas for the treatment of Type 1 Diabetes Mellitus. More specifically, insulin stimulating peptides were conjugated to a polymeric backbone and co-encapsulated with pancreatic islets in an alginate-poly-L-lysine microcapsule, and their potential to increase insulin output in response to elevated glucose levels was examined. Although this work is not directly tied to the main body of the dissertation, it is included in this dissertation for completeness.

1.7. Specific Aims

Combinational therapy is becoming more and more prevalent as a go-to method for treating many diseases including cancer [256-258]. Administering several medications together is often more beneficial than monotherapy approaches [259-261]. However, ensuring the delivery of all therapeutics to a target location is difficult, decreasing the overall effectiveness of the treatment. Co-delivery of agents simultaneously to a specific location would greatly enhance the efficacy of combinational therapy, and could mitigate side effects and potentially decrease the amount of drug necessary for treatment [262, 263]. I proposed to investigate the targeted delivery of two therapeutic agents simultaneously to the nucleus of a cell using a polymeric micelle carrier system.

The micelle system will be a core-shell structure composed of the copolymer poly(DL-lactide-co-glycolide) and branched polyethyleneimine (PLGA-*b*-bPEI). The PLGA block will form the hydrophobic core region and the PEI block will form the hydrophilic

corona or shell region. Micelles should be internalized into the cell by endocytosis. PEI is well-known to have proton-buffering capacity and aid in endosomal escape due to the proton-sponge effect. Therapeutic agents can be loaded either into the hydrophobic core or bound to the hydrophilic shell of the micelle. Two different model therapeutic agents will be investigated: firefly luciferase reporter gene as a model gene drug, and dexamethasone as a model chemical drug. Dexamethasone is known to dilate nuclear pore complexes [264-266], and will be incorporated into the micelle core to facilitate increased nuclear delivery. Dexamethasone will be loaded into the core of the micelle during micelle formation by dialysis methods. The luciferase gene will be incubated with the drug-loaded micelles, allowing the gene to interact with the PEI corona layer and bind through electrostatic interactions, forming a micelle-gene complex. Thus, this core-shell micelle structure can be used to simultaneously deliver different kinds of therapeutics to a target site.

Aim 1: Demonstrate that two therapeutic agents can be loaded simultaneously into a PLGA-*b*-bPEI polymeric micelle carrier.

a) Construction of the copolymer micelle system – The PLGA-*b*-bPEI copolymer will be synthesized, characterized and used to create the micelle carrier system for this study.
b) Model therapeutic agents – The luciferase gene will serve as a model gene drug and the glucocorticoid analog dexamethasone will serve as the model chemical drug for this micelle carrier delivery system.

c) Drug loading – Dexamethasone will be loaded into the micelle core during micelle formation by dialysis. PLGA-*b*-bPEI polymer and dexamethasone will be mixed together in dimethyl sulfoxide and dialyzed against water to form the drug-loaded micelles. The luciferase gene will be loaded after micelle formation is complete. Micelles will be mixed with the gene and incubated together for 30 minutes to allow for complexation to occur. These gene-micelle complexes will then be used for further studies.

Aim 2: Prove that the polymeric carrier system co-loaded with dexamethasone and a gene is able to accumulate inside the nucleus of the cell and release its drug load within the nucleus and assess its potential efficacy by evaluating gene expression.

a) Confirm nuclear accumulation of the carrier system – The presence of dexamethasone is known to have a dilatory effect on the nuclear pore complex which could aid in accumulation of the micelle carrier inside the nucleus. Once inside, gene transfection should occur. Accumulation of the carrier in the nucleus can be confirmed visually using fluorescence staining techniques and imaged using confocal microscopy.

b) Confirm drug release within the nucleus – Gene drug delivery can be confirmed by evaluating gene expression. Comparing gene expression from micelles with dexamethasone versus without dexamethasone will indicate whether the drug is releasing, as drug release will increase gene transfection and subsequent gene expression.

c) Confirm gene expression in breast cancer cells – Uptake of the micelle system should result in transcription and expression of the luciferase gene, which can be measured by a standard reporter gene assay normalized to total protein content in the cell.

1.8. References

[1] Culver K. Gene Therapy - A Handbook for Physicians. New York: Mary Ann Liebert, Inc., 1994.

[2] Goncalves MA. A concise peer into the background, initial thoughts and practices of human gene therapy. Bioessays 2005;27(5):506-517.

[3] Cotrim AP, Baum BJ. Gene therapy: some history, applications, problems, and prospects. Toxicol Pathol 2008;36(1):97-103.

[4] Friedmann T, Roblin R. Gene therapy for human genetic disease? Science 1972;175(25):949-955.

[5] Szybalska EH, Szybalski W. Genetics of human cell lines, IV. DNA-mediated heritable transformation of a biochemical trait. Proc Natl Acad Sci USA 1962;48:2026-2034.

[6] Borenfreund E, Bendich A. A study of the penetration of mammalian cells by deoxyribonucleic acids. J Biophys Biochem Cyt 1961;9(1):81-91.

[7] Kay ERM. Incorporation of deoxyribonucleic acid by mammalian cells *in vitro*. Nature 1961;191:387-388.

[8] Rabotti GF. Incorporation of DNA into a mouse tumor *in vivo* and in vitro. Exp Cell Res 1963;31:562-565.

[9] Bradley TR, Roosa RA, Law L. DNA transformation studies with mammalian cells in culture. J Cell Comp Physiol 1962;60:127-137.

[10] Majumdar A, Bose SK. DNA mediated genetic transformation of a human cancerous cell line culture *in vitro.* Br J Cancer 1968;22(3):603-613.

[11] Bendich A, Borenfreund E, Honda Y. DNA-induced heritable alteration of mammalian cells. Amsterdam: North-Holland Publishing Company, 1971. p. 80-87.

[12] Hill M, Huppert J. Fate of exogenous mouse DNA in chicken fibroblasts in vitro: non-conservative perservation. Biochim Biophys Acta 1970;213(1):26-35.

[13] Aposhian HV, Qasba PK, Osterman JV, Waddell A. Polyoma pseudovirions: An experimental model for the development of DNA for gene therapy. Fed Proc 1972;31(4):1310-1314.

[14] Aposhian HV. The use of DNA for gene therapy: the need, experimental approach, and implications. Perspect Biol Med 1970;14(1):98-108.

[15] Friedmann T. In vitro reassemmbly of shell-like particles from disrupted polyoma virus. Proc Natl Acad Sci USA 1971;68(10):2574-2578.

[16] Tatum EL. Molecular biology, nucleic acids and the future of medicine. Perspect Biol Med 1966;10(1):19-32.

[17] Sinsheimer R. The prospect for designed genetic change. Am Sci 1969;57(1):134-142.

[18] The Prospects of Gene Therapy. Bethesda: Fogarty International Center, NIH, 1972.

[19] Anderson WF. Human Gene therapy. Science 1992;256(5058):808-813.

[20] Mulligan RC, Howard BH, Berg P. Synthesis of rabbit beta-globin in cultured monkey kidney cells following infection with a SV40 beta-globin recombinant genome. Nature 1979;277(5692):108-114.

[21] Green MR, Treisman R, Maniatis T. Transcriptional activation of cloned human beta-globin genes by viral immediate-early gene products. Cell 1982;35(1):137-148.

[22] Mulligan RC, Berg P. Selection for animal cells that express the Escherichia coli gene for xanthine-guanine phosphoribosyltransferase. Proc Natl Acad Sci USA 1981;78(4):2072-2076.

[23] Mulligan RC, Berg P. Factors governing the expression of a bacterial gene in mammalian cells. Mol Cell Biol 1981;1(5):449-459.

[24] Temin HM. Mechanism of cell transformation by RNA tumor viruses. Annu Rev Microbiol 1971;25:609-648.

[25] Temin HM. The DNA provirus hypothesis: the establishment and implications of RNA-directed DNA synthesis. Science 1976;192(4244):1075-1080.

[26] Temin HM, Mizutani S. RNA-directed DNA polymerase in virions of Rous sarcoma virus. Nature 1970;226(5252):1211-1213.

[27] Baltimore D. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature 1970;226(5252):1209-1211.

[28] Shimotohno K, Temin HM. Formation of infectious progeny virus after instertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. Cell 1981;26(1 Pt 1):67-77.

[29] Wei C, Gibson M, Spear PG, Scolnick EM. Construction and isolation of a transmissible retrovirus containing the src gene from Harvey Murine Sarcoma Virus and the thymidine kinase gene from herpes simplex virus type 1. J Virol 1981;39(3):935-944.

[30] Tabin CJ, Hoffmann JW, Goff SP, Weinberg RA. Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex thymidine kinase gene. Mol Cell Biol 1982;2(4):426-436.

[31] Doehmer J, Barinaga M, Vale W, Rosenfeld MG, Verma IM, Evans RM. Introduction of rat growth hormone into mouse fibroblasts via a retroviral DNA vector. Proc Natl Acad Sci USA 1982;79(7):2268-2272.

[32] Miller AD, Law MF, Verma IM. Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. Mol Cell Biol 1985;5(3):431-437.

[33] Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 1986;6(8):2895-2902.

[34] Mann R, Mulligan RC, Baltimore D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 1983;33(1):153-159.

[35] Jolly DJ, Okayama H, Berg P, Esty AC, Filpula D, Bohlen P, et al. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyl transferase. Proc Natl Acad Sci USA 1983;80(2):477-481.

[36] Miller AD, Jolly DJ, Friedmann T, Verma IM. A transmissible retrovirus expressing human hypoxanthine phosphoribosyltrasnferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. Proc Natl Acad Sci USA 1983;80(15):4709-4713.

[37] Willis RC, Jolly DJ, Miller AD, Plent MM, Esty AC, Anderson PJ, et al. Partial phenotypic correction of human Lesch-Nyhan (hypoxanthine-guanine phosphoribosyltransferase-deficient) lymphoblasts with a transmissible retroviral vector. J Biol Chem 1984;259(12):7842-7849.

[38] Rogers S, Lowenthal A, Terheggen HG, Columbo JP. Induction of arginase activity with the Shope papilloma virus in tissue culture cells from an argininemic patient. J Exp Med 1973;137(4):1091-1096.

[39] Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, R. M, et al. Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. New Eng J Med 1990;323(9):570-578.

[40] Palmer TD, Thompson AR, Miller AD. Production of human factor IX in animals by genetically modified skin fibroblasts: Potential therapy for hemophilia B. Blood 1989;73(2):438-445.

[41] Axelrod JH, Read MS, Brinkhous KM, Verma IM. Phenotypic correction of factor IX deficiency in skin fibroblasts of hemophilic dogs. Proc Natl Acad Sci USA 1990;87(13):5173-5177.

[42] Roman M, Axelrod JH, Dai Y, Naviaux RK, Friedmann T, Verma IM. Circulating human or canine factor IX from retrovirally transduced primary myoblasts and established myoblast cell lines grafted into murine skeletal muscle. Somat Cell Mol Genet 1992;18(3):247-258.

[43] Miyanohara A, Johnson PA, Elam RL, Dai Y, Witztum JL, Verma IM, et al. Direct gene transfer to the liver with herpes simplex virus type 1 vectors: Transient production of physiologically relevant levels of circulating factor IX. New Biol 1992;4(3):238-246.

[44] Yao SN, Wilson JM, Nabel EG, Kurachi S, Hachiya HL, Kurachi K. Expression of human factor IX in rat capillary endothelial cells: Toward somatic gene therapy for hemophilia B. Proc Natl Acad Sci USA 1991;88(18):8101-8105.

[45] Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen HJ. Adenosine-deaminase deficiency in two patients with severely imparied cellular immunity. Lancet 1972;2(7786):1067-1069.

[46] van Beusechem VW, Kukler A, Einerhand MP, Bakx TA, van der Eb AJ, van Bekkum DW, et al. Expression of human adenosine deaminase in mice transplanted with hemopoietic stem cells infected with amphotropic retroviruses. J Exp Med 1990;172(3):729-736.

[47] Miyanohara A, Sharkey MF, Witztum JL, Steinberg D, Friedmann T. Efficient expression of retroviral vector-transduced human low density lipoprotein (LDL) receptor in LDL receptor-deficient rabbit fibroblasts in vitro. Proc Natl Acad Sci USA 1988;85(17):6538-6542.

[48] Edelstein ML, Abedi MR, Wixon J, Edelstein RM. Gene therapy clinical trials worldwide 1989-2004-an overview. J Gene Med 2004;6(6):597-602.

[49] Feng LR, Maguire-Zeiss KA. Gene therapy in Parkinson's disease: rationale and current status. CNS Drugs 2010;24(3):177-192.

[50] Jorgensen C, Apparailly F. Prospects for gene therapy in inflammatory arthritis. Best Pract Res Clin Rheumatol 2010;24(4):541-552.

[51] Poller W, Hajjar R, Schultheiss HP, Fechner H. Cardiac-targeted delivery of regulatory RNA molecules and genes for the treatment of heart failure. Cardiovasc Res 2010;86(3):353-364.

[52] DiPrimio N, McPhee SW, Samulski RJ. Adeno-associated virus for the treatment of muscle diseases: toward clinical trials. Curr Opin Mol Ther 2010;12(5):553-560.

[53] Wang Y, Asakawa A, Inui A, Kosai K. Leptin gene therapy in the fight against diabetes. Expert Opin Biol Ther 2010;10(10):1405-1414.

[54] Vachani A, Moon E, Wakeam E, Albelda SM. Gene therapy for mesothelioma and lung cancer. Am J Respir Cell Mol Biol 2010;42(4):385-393.

[55] Pedersini R, Vattemi E, Claudio PP. Adenoviral gene therapy in high-grade malignant glioma. Drug News Perspect 2010;23(6):368-379.

[56] Bexell D, Scheding S, Bengzon J. Toward brain tumor gene therapy using multipotent mesenchymal stromal cell vectors. Mol Ther 2010;18(6):1067-1075.

[57] Quetglas JI, Ruiz-Guillen M, Aranda A, Casales E, Bezunartea J, Smerdou C. Alphavirus vectors for cancer therapy. Virus Res 2010;153(2):179-196.

[58] Pfeifer A, Verma IM. Gene Therapy: Promises and Problems. Annu Rev Genomics Hum Genet 2001;2:177-211.

[59] Seth P. Vector-mediated cancer gene therapy: an overview. Cancer Biol Ther 2005;4(5):512-517.

[60] Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, et al. T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. Science 1995;270(5235):475-480.

[61] Aiuti A, Roncarolo MG. Hematology: Ten years of gene therapy for primary immune deficiencies. Am Soc Hematol Educ Program 2009:682-689.

[62] Eden ER, Patel DD, Sun XM, Burden JJ, Themis M, Edwards M, et al. Restoration of LDL receptor function in cells from patients with autosomal recessive hypercholesterolemia by retroviral expression of ARH1. J Clin Invest 2002;110(11):1695-1702.

[63] Liu L, Wang S, Shan B, Sang M, Liu S, Wang G. Advances in viral-vector systemic cytokine gene therapy against cancer. Vaccine 2010;28(23):3883-3887.

[64] Rochlitz CF. Gene therapy of cancer. Swiss Med Wkly 2001;131:4-9.

[65] Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al. LMO2- associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003;302(5644):415-419.

[66] Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrangeli A, Hay JG, et al. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat Genet 1994;8(1):42-51.

[67] Seth P. Adenoviral vectors. Adv Exp Med Biol 2000;465:13-22.

[68] van der Most RG, Robinson BW, Nelson DJ. Gene therapy for malignant mesothelioma: beyond the infant years. Cancer Gene Ther 2006;13(10):897-904.

[69] Morse MA. Virus-based therapies for colon cancer. Expert Opin Biol Ther 2005;5(12):1627-1633.

[70] Dani SU. The challenge of vector development in gene therapy. Braz J Med Biol Res 1999;32(2):133-145.

[71] Berns KI. Parvoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott-Raven, 1996. p. 2173-2197.

[72] Verma IM, Somia N. Gene therapy- promises, problems and prospects. Nature 1997;389(6648):239-242.

[73] Flotte T, Carter B, Conrad C, Guggino W, Reynolds T, Rosenstein B, et al. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. Hum Gene Ther 1996;7(9):1145-1159.

[74] Flotte TR, Zeitlin PL, Reynolds TC, Heald AE, Pedersen P, Beck S, et al. Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: A two-part clinical study. Hum Gene Ther 2003;14(11):1079-1088.

[75] Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000;24(3):257-261.

[76] Fiandaca MS, Bankiewicz KS. Gene therapy for Parkinson's disease: from non-human primates to humans. Curr Opin Mol Ther 2010;12(5):519-529.

[77] Flotte TR. Gene Therapy: The first two decades and the current state-of-the-art. J Cell Physiol 2007;213(2):301-305.

[78] Zufferey R, Dull T, Mandel RJ, Bukovsky AQ, D., Naldini L, Trono D. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 1998;72(12):9873-9880.

[79] Abordo-Adesida E, Follenzi A, Barcia C, Sciascia S, Castro MG, Naldini L, et al. Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivector immune responses. Hum Gene Ther 2005;16(6):741-751.

[80] Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther 1999;6(9):1574-1583.

[81] Mingozzi F, Maus MV, Hui DJ, Sabatino DE, Murphy SL, Rasko JE, et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat Med 2007;13(4):419-422.

[82] Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996;272(5259):263-267.

[83] Persons DA. Hematopoietic stem cell gene transfer for the treatment of hemoglobin disorders. Hematology Am Soc Hematol Educ Program 2009:690-697.

[84] Azzouz M, Kingsman SM, Mazarakis ND. Lentiviral vectors for treating and modeling human CNS disorders. J Gene Med 2004;6(9):951-962.

[85] Breckpot K, Emeagi PU, Thielemans K. Lentiviral vectors for anti-tumor immunotherapy. Curr Gene Ther 2008;8(6):438-448.

[86] Lemiale F, Korokhov N. Lentiviral vectors for HIV disease prevention and treatment. Vaccine 2009;27(25-26):3443-3449.

[87] Epstein AL. HSV-1-derived amplicon vectors: recent technological improvements and remaining difficulties--a review. Mem Inst Oswaldo Cruz 2009;104(3):399-410.

[88] Watanabe D. Medical application of herpes simplex virus. J Dermatol Sci 2009;57(2):75-82.

[89] Marconi P, Argnani R, Epstein AL, Manservigi R. HSV as a vector in vaccine development and gene therapy. Adv Exp Med Biol 2009;655:118-144.

[90] Liu RB, Rabkin SD. Oncolytic herpes simplex virus vectors for the treatment of human breast cancer. Chin Med J 2005;118(4):307-312.

[91] Wolfe D, Mata M, Fink DJ. A human trial of HSV-mediated gene transfer for the treatment of chronic pain. Gene Ther 2009;16(4):455-460.

[92] Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab 2003;80(1-2):148-158.

[93] Philips AJ. The challenge of gene therapy and DNA delivery. J Pharm Pharmacol 2001;53(9):1169-1174.

[94] Thomas D, Suthanthiran M. Optimal modes and targets of gene therapy in transplantation. Immunol Rev 2003;196(161-75).

[95] del Pozo-Rodriguez A, Solinis MA, Gascon AR, Pedraz JL. Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. Eur J Pharm Biopharm 2009;71(2):181-189.

[96] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247(4949 Pt 1):1465-1468.

[97] Li S, Ma Z. Nonviral gene therapy. Curr Gene Ther 2001;1(2):201-226.

[98] Takeshita S, Isner JM. Peripheral angiogenesis: therapeutic angiogenesis for peripheral vascular occlusive disease. Curr Interv Cardiol Rep 1999;1(2):147-156.

[99] Danko I, Wolff JA. Direct gene transfer into muscle. Vaccine 1994;12(16):1499-1502.

[100] He YK, Lui VW, Baar J, Wang L, Shurin M, Almonte C, et al. Potentiation of E7 antisense RNA-induced antitumor immunity by codelivery of IL-12 gene in HPV16 DNA-positive mouse tumor. Gene Ther 1998;5(11):1462-1471.

[101] Lasic DD. Doxorubicin in sterically stabilized liposomes. Nature 1996;380(6574):561-562.

[102] Fraley R, Subramani S, Berg P, Papahadjopoulos D. Introduction of liposome-encapsulated SV40 DNA into cells. J Biol Chem 1980;255(21):10431-10435.

[103] Wang CY, Huang L. pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse. Proc Natl Acad Sci USA 1987;84(22):7851-7855.

[104] Schäfer J, Höbel S, Bakowsky U, Aigner A. Liposome-polyethylenimine complexes for enhanced DNA and siRNA delivery. Biomaterials 2010;31(26):6892-6900.

[105] Hirashima N, Minatani K, Hattori Y, Ohwada T, Nakanishi M. Anchoring cationic amphiphiles for nucleotide delivery: significance of DNA release from cationic liposomes for transfection. Biol Pharm Bull 2007;30(6):1117-1122.

[106] Egilmez NK, Iwanuma Y, Bankert RB. Evaluation and optimization of different cationic liposome formulations for in vivo gene transfer. Biochem Biophys Res Commun 1996;221(1):169-173.

[107] Yoshimura K, Rosenfeld MA, Nakamura H, Scherer EM, Pavirani A, Lecocq JP, et al. Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after in vivo intratracheal plasmid-mediated gene transfer. Nucleic Acids Res 1992;20(12):3233-3240.

[108] McDonald RJ, Liggitt HD, Roche L, Nguyen HT, Pearlman R, Raabe OG, et al. Aerosol delivery of lipid:DNA complexes to lungs of rhesus monkeys. Pharm Res 1998;15(5):671-679.

[109] Yu D, Matin A, Xia W, Sorgi F, Huang L, Hung MC. Liposome-mediated in vivo E1A gene transfer suppressed dissemination of ovarian cancer cells that overexpress HER-2/neu. Oncogene 1995;11(7):1383-1388.

[110] Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly (ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. FEBS Lett 1997;400(2):233-237.

[111] Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, et al. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. Proc Natl Acad Sci USA 1993;90(23):11307-11311.

[112] Hui KM, Ang PT, Huang L, Tay SK. Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA-liposome complexes. Gene Ther 1997;4(8):783-790.

[113] Stopeck AT, Jones A, Hersh EM, Thompson JA, Finucane DM, Gutheil JC, et al. Phase II study of direct intralesional gene transfer of allovectin-7, an HLA-B7/beta2-microglobulin DNA-liposome complex, in patients with metastatic melanoma. Clin Cancer Res 2001;7(8):2285-2291.

[114] Yoshida J, Mizuno M. Clinical gene therapy for brain tumors. Liposomal delivery of anticancer molecule to glioma. J Neurooncol 2003;65(3):261-267.

[115] Boorjian SA, Milowsky MI, Kaplan J, Albert M, Cobham MV, Coll DM, et al. Phase 1/2 clinical trial of interferon alpha2b and weekly liposome-encapsulated all-trans retinoic acid in patients with advanced renal cell carcinoma. J Immunother 2007;30(6):655-662.

[116] Antón A, Ruiz A, Seguí MA, Calvo L, Muñoz ML, J., Sancho F, et al. Phase I clinical trial of liposomal-encapsulated doxorubicin citrate and docetaxel, associated with trastuzumab, as neo-adjuvant treatment in stages II and IIIA, HER2-overexpressing breast cancer patients. GEICAM 2003-03 study. Ann Oncol 2009;20(3):454-459.

[117] Wolff AC, Wang M, Li H, Pins MR, Pretorius FJ, Rowland KM, et al. Phase II trial of pegylated liposomal doxorubicin plus docetaxel with and without trastuzumab in metastatic breast cancer: Eastern Cooperative Oncology Group trial E3198. Breast Cancer Res Treat 2010;121(1):111-120.

[118] Sorscher EJ, Logan JJ, Frizzell RA, Lyrene RK, Bebok Z, Dong JY, et al. Gene therapy for cystic fibrosis using cationic liposome mediated gene transfer: a phase I trial of safety and efficacy in the nasal airway. Hum Gene Ther 1994;5(10):1259-1277.

[119] Caplen NJ, Alton EWFW, Middleton PG, Dorin JR, Stevenson BJ, Gao X, et al. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nature Med 1995;1(1):39-46.

[120] McLachlan G, Ho LP, Davidson-Smith H, Samways J, Davidson H, Stevenson BJ, et al. Laboratory and clinical studies in support of cystic fibrosis Gene Therapy using pCMV-CFTR-DOTAP. Gene Ther 1996;3(12):1113-1123.

[121] Gill DR, Southern KW, Mofford KA, Seddon T, Huang L, Sorgi F, et al. A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Ther 1997;4(3):199-209.

[122] Wu GY, Wu CH. Receptor-mediated gene delivery and expression in vivo. J Biol Chem 1988;263(29):14621-14624.

[123] Lim YB, Choi YH, Park JS. A self-destroying polycationic polymer: biodegradable poly(4-hydroxy-L-proline ester). J Am Chem Soc 1999;121(24):5633-5639.

[124] Putnam D, Langer R. Poly(4-hydroxy-L-proline ester): low temperature polycondensation and plasmid DNA complexation. Macromol 1999;32(11):3658-3662.

[125] Lim YB, Kim CH, Kim K, Kim SW, Park JS. Development of a safe gene delivery system using biodegradable polymer, poly[α-(4-aminobutyl)-L-glycolic acid]. J Am Chem Soc 2000;122(27):6524-6525.

[126] Lim YB, Han SO, Kong HU, Lee Y, Park JS, Jeong B, et al. Biodegradable polyester, poly[α-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. Pharm Res 2000;17(7):811-816.

[127] Zhong Z, Song Y, Engbersen JFJ, Lok MC, Hennink WE, Feijen J. A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. J Control Release 2005;109(1-3):317-329.

[128] Wagner E, Ogris M, Zauner W. Polylysine-based transfection systems utilizing receptor-mediated delivery. Adv Drug Deliv Rev 1998;30(1-3):97-113.

[129] Lee H, Jeong JH, Park TG. PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. Journal of Controlled Release 2002;79(1-3):283-291.

[130] Jeong JH, Park TG. Poly(L-lysine)-g-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J Control Release 2002;82(1):159-166.

[131] Bikram M, Ahn CH, Chae SY, Lee M, Yockman J, Kim SW. Biodegradable poly(ethylene glycol)-co-poly(L-lysine)-g-histidine multiblock copolymers for nonviral gene delivery. Macromol 2004;37(5):1903-1916.

[132] Bikram M, Lee M, Chang CW, Janát-Amsbury MM, Kern SE, Kim SW. Long-circulating DNA-complexed biodegradable multiblock copolymers for gene delivery: degradation profiles and evidence of dysopsonization. J Control Release 2005;103(1):221-233.

[133] Kakizawa Y, Harada A, Kataoka K. Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly(ethylene glycol)-block–poly(L-lysine): a potential carrier for systemic delivery of antisense DNA. Biomacromolecules 2001;2(2):491-497.

[134] Funhoff AM, van Nostrum CF, Janssen AP, Fens MH, Crommelin DJ, Hennink WE. Polymer side-chain degradation as a tool to control the destabilization of polyplexes. Pharm Res 2004;21(1):170-176.

[135] Ríhová B, Kubácková K. Clinical implications of N-(2-hydroxypropyl) methacrylamide copolymers. Curr Pharm Biotechnol 2003;4(5):311-322.

[136] Luten J, Akeroyd N, Funhoff A, Lok MC, Talsma H, Hennink WE. Methacrylamide polymers with hydrolysis-sensitive cationic side groups as degradable gene carriers. Bioconjug Chem 2006;17(4):1077-1084.

[137] de Wolf HK, Luten J, Snel CJ, Storm G, Hennink WE. Biodegradable, cationic methacrylamide based polymers for gene delivery to ovarian cancer cells in mice. Mol Pharm 2008;5(2):349-357.

[138] Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 1995;92(16):7297-7301.

[139] Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Release 1999;60(2-3):149-160.

[140] Fischer D, Bieber T, Li Y, Elsässer HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res 1999;16(8):1273-1279.

[141] Ahn CH, Chae SY, Bae YH, Kim SW. Biodegradable poly(ethylenimine) for plasmid DNA delivery. J Control Release 2002;80(1-3):273-282.

[142] Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther 1997;4(8):823-832.

[143] Toncheva V, Wolfert MA, Dash PR, Oupicky D, Ulbrich K, Seymour LW, et al. Novel vectors for gene delivery formed by self-assembly of DNA with poly (L-lysine) grafted with hydrophilic polymers. Biochim Biophys Acta 1998;1380(3):354-368.

[144] Park SY, Harries D, Gelbart WM. Topological defects and the optimum size of DNA condensates. Biophys J 1998;75(2):714-720.

[145] Perales JC, Grossmann GA, Molas M, Liu G, Ferkol T, Harpst J, et al. Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. J Biol Chem 1997;272(11):7398-7407.

[146] Leong KW. Biopolymer-DNA nanospheres. In: Huang L, Hung M-C, Wagner E, editors. Nonviral vectors for Gene Therapy. San Diego: Academic Press, 1999. p. 267-287.

[147] Ravi Kumar MN. Nano and microparticles as controlled drug delivery devices. J Pharm Pharm Sci 2000;3(2):234-258.

[148] Wang D, Robinson DR, Kwon GS, Samuel J. Encapsulation of plasmid DNA in biodegradable poly(D, L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. J Control Release 1999;57(1):9-18.

[149] De Smedt SC, Demeester J, Hennink WE. Cationic polymer based gene delivery systems. Pharm Res 2000;17(2):113-126.

[150] Addison T, Cayre OJ, Biggs S, Armes SP, York D. Incorporation of block copolymer micelles into multilayer films for use as nanodelivery systems. Langmuir 2008;24(23):13328-13333.

[151] Kang HC, Bae YH. pH-Tunable endosomolytic oligomers for enhanced nucleic acid delivery. Adv Funct Mater 2007;17(8):1263-1272.

[152] Kakizawa Y, Kataoka K. Block copolymer micelles for delivery of gene and related compounds. Adv Drug Deliv Rev 2002;54(2):203-222.

[153] Davis ME, Chen Z, Shin D. Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov 2008;7(9):771-782.

[154] Kim D, Lee ES, Oh KT, Gao ZG, Bae YH. Doxorubicin-loaded polymeric micelles overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. Small 2008;4(11):2043-2050.

[155] Jeong YI, Seo SJ, Park IK, Lee HC, Kang IC, Akaike T, et al. Cellular recognition of paclitaxel-loaded polymeric nanoparticles composed of poly(gamma-benzyl L-glutamate) and poly(ethylene glycol) diblock copolymer endcapped with galactose moiety. Int J Pharm 2005;296(1-2):151-161.

[156] Louise C. Nonviral vectors. Methods Mol Biol 2006;333:201-226.

[157] Vasir JK, Labhasetwar V. Polymeric nanoparticles for gene delivery. Expert Opin Drug Deliv 2006;3(3):325-344.

[158] Dubruel P, Schacht EH. Vinyl polymers as non-viral gene delivery carriers: current status and prospects. Macromol Biosci 2006;6(10):789-810.

[159] Pietersz GA, Tang CK, Apostolopoulos V. Structure and design of polycationic carriers for gene delivery. Mini Rev Med Chem 2006;6(12):1285-1298.

[160] Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv Drug Deliv Rev 2002;54(5):715-758.

[161] Brown MD, Schatzlein AG, Uchegbu IF. Gene delivery with synthetic (non-viral) carriers. Int J Pharm 2001;229(1-2):1-21.

[162] Yang TF, Chin WK, Cherng JY, Shau MD. Synthesis of novel biodegradable cationic polymer: N,N-diethylethylenediamine polyurethane as a gene carrier. Biomacromolecules 2004;5(5):1926-1932.

[163] Shau MD, Tseng SJ, Yang TF, Cherng JY, Chin WK. Effect of molecular weight on the transfection efficiency of novel polyurethane as a biodegradable gene vector. J Biomed Mater Res A 2006;77(44):736-746.

[164] Wang J, Mao HQ, Leong KW. A novel biodegradable gene carrier based on polyphosphoester. J Am Chem Soc 2001;123(38):9480-9481.

[165] Wang J, Huang SW, Zhang PC, Mao HQ, Leong KW. Effect of side-chain structures on gene transfer efficiency of biodegradable cationic polyphosphoesters. Int J Pharm 2003;265(1-2):75-84.

[166] Luten J, van Steenis JH, van Someren R, Kemmink J, Schuurmans-Nieuwenbroek NM, Koning GA, et al. Water-soluble biodegradable cationic polyphosphazenes for gene delivery. J Control Release 2003;89(3):483-497.

[167] Wu GY, Wu CH. Evidence for targeted gene delivery to HepG2 hepatoma cells in vitro. Biochemistry 1988;27(3):887-892.

[168] Wagner E, Zenke M, Cotten M, Beug H, Birnstiel ML. Transferrin-polycation conjugates as carriers for DNA uptake into cells. Proc Natl Acad Sci USA 1990;87(9):3410-3414.

[169] Cotten M, Längle-Rouault F, Kirlappos H, Wagner E, Mechtler K, Zenke M, et al. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. Proc Natl Acad Sci USA 1990;87(11):4033-4037.

[170] Trubetskoy VS, Torchilin VP, Kennel SJ, Huang L. Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. Bioconjug Chem 1992;3(4):323-327.

[171] Citro G, Ginobbi P, Szczylik C. Receptor-mediated oligodeoxynucleotides delivery by estradiol and folic acid polylysine conjugates. Cytotechnology 1993;11 Suppl 1:S30-34.

[172] Strydom S, Van Jaarsveld P, Van Helden E, Ariatti M, Hawtrey A. Studies on the transfer of DNA into cells through use of avidin-polylysine conjugates complexed to biotinylated transferrin and DNA. J Drug Target 1993;1(2):165-174.

[173] Sikes ML, O'Malley BWJ, Finegold MJ, Ledley FD. In vivo gene transfer into rabbit thyroid follicular cells by direct DNA injection. Hum Gene Ther 1994;5(7):837-844.

[174] Erbacher P, Bousser MT, Raimond J, Monsigny M, Midoux P, Roche AC. Gene transfer by DNA/glycosylated polylysine complexes into human blood monocyte-derived macrophages. Hum Gene Ther 1996;7(6):721-729.

[175] Koh JJ, Ko KS, Lee M, Han S, Park JS, Kim SW. Degradable polymeric carrier for the delivery of IL-10 plasmid DNA to prevent autoimmunie insulitis of NOD mice. Gene Ther 2000;7(24):2099-2104.

[176] Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Deliv 2005;4(581-93).

[177] Dennig J, Duncan E. Gene transfer into eukaryotic cells using activated polyamidoamine dendrimers. Rev Mol Biotech 2002;90(3-4):339-347.

[178] Tang MX, Redemann T, Szoka FC. In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconjug Chem 1996;7(6):703-714.

[179] Rafty LA, Khachigian LM. Zinc finger transcription factors mediate high constitutive platelet-derived growth factor-B expression in smooth muscle cells derived from aortae of newborn rats. J Biol Chem 1998;273(10):5758-5764.

[180] Daniels RH, Hall PS, Bokoch GM. Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC-12 cells. EMBO J 1998;17(3):754-764.

[181] Albritton LM. Efficient transfection of fibroblast and epithelial cells using an activated dendrimer reagent. J NIH Res 1997;9:52.

[182] Bielinska A, Kukowska-Latallo JF, Johnson J, Tomalia DA, Baker JR. Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. Nucleic Acids Res 1996;24(11):2176-2182.

[183] Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA, Baker JR. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. Proc Natl Acad Sci USA 1996;93(10):4897-4902.

[184] Gao Y, Gao G, He Y, Liu T, Qi R. Recent advances of dendrimers in delivery of genes and drugs. Mini Rev Med Chem 2008;8(9):889-900.

[185] Tanaka S, Iwai M, Harada Y, Morikawa T, Muramatsu A, Mori T, et al. Targeted killing of carcinoembryonic antigen (CEA)-producing cholangiocarcinoma cells by polyamido amine dendrimer-mediated transfer of an Epstein-Barr virus (EBV)-based plasmid vector carrying the CEA promoter. Cancer Gene Ther 2000;7(9):1241-1250.

[186] Rudolph C, Lausier J, Naundorf S, Muller RH, Rosenecker J. *In vivo* gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. J Gene Med 2000;2(4):269-278.

[187] Harada Y, Iwai M, Tanaka S, Okanoue T, Kashima K, Maruyama-Tabata H, et al. Highly efficient suicide gene expression in hepatocellular carcinoma cells by Epstein-Barr virusbased plasmid vectors combined with polyamidoamine dendrimer. Cancer Gene Ther 2000;7(1):27-36.

[188] Cherng JY, van de Wetering P, Talsma H, Crommelin DJA, Hennink WE. Effect of size and serum proteins on transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. Pharm Res 1996;13(7):1038-1042.

[189] van de Wetering P, Cherng JY, Talsma H, Crommelin DJA, Hennink WE. 2-(dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. J Control Release 1998;53(1-3):145-153.

[190] Verbaan FJ, Oussoren C, Snel CJ, Crommelin DJA, Hennink WE, Storm G. Steric stabilization of poly(2-(dimethylamino)ethyt methacrytate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. J Gene Med 2004;6(1):64-75.

[191] You YZ, Manickam DS, Zhou QH, Oupicky D. Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. J Control Release 2007;122(3):217-225.

[192] Convertine AJ, Benoit DSW, Duvall CL, Hoffman AS, Stayton PS. Development of a novel endosomolytic diblock copolymer for siRNA delivery. J Control Release 2009;133(3):221-229.

[193] Zhang P, Liu W. ZnO QD@PMAA-co-PDMAEMA nonviral vector for plasmid DNA delivery and bioimaging. Biomaterials 2010;31(11):3087-3094.

[194] Zhu C, Jung S, Luo S, Meng F, Zhu X, Park TG, et al. Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA-PCL-PDMAEMA triblock copolymers. Biomaterials 2010;31(8):2408-2416.

[195] Gao C, Liu M, Chen J, Chen C. pH- and temperature-responsive P(DMAEMA-GMA)-alginate semi-IPN hydrogels formed by radical and ring-opening polymerization for aminophylline release. J Biomater Sci Polym Ed 2011;Epub April 21, 2011.

[196] Khan AR, Forgo P, Stine KJ, D'Souza VT. Methods for selective modifications of cyclodextrins. Chem Rev 1998;98(5):1977-1996.

[197] Uekama K, Hirayama F, Irie I. Cyclodextrin drug carrier systems. Chem Rev 1998;98(5):2045-2076.

[198] Challa R, Ahuja A, Ali J, Khar RK. Cyclodextrins in drug delivery: an updated review. AAPS PharmSciTech 2005;6(2):E329-357.

[199] Villalonga R, Cao R, Fragoso A. Supramolecular chemistry of cyclodextrins in enzyme technology. Chem Rev 2007;107(7):3088-3116.

[200] Mellet CO, Fernández JM, Benito JM. Cyclodextrin-based gene delivery systems. Chem Soc Rev 2011;40(3):1586-1608.

[201] Habus I, Zhao Q, Agrawal S. Synthesis, hybridization properties, nuclease stability, and cellular update of the oligonucleotide-amino-beta-cyclodextrins and adamantane conjugates. Bioconjug Chem 1995;6(4):327-331.

[202] Redenti E, Pietra C, Gerloczy A, Szente L. Cyclodextrins in oligonucleotide delivery. Adv Drug Deliv Rev 2001;53(2):235-244.

[203] Freemann DJ, Niven RW. The influence of sodium glycocholate and other additives on the in vivo transfection of plasmid DNA in the lungs. Pharm Res 1996;13(2):202-209.

[204] Kichler A, Leborgne C, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. J Gene Med 2001;3(2):135-144.

[205] Kichler A. Gene transfer with modified polyethylenimines. J Gene Med 2004;6 Suppl 1:S3-10.

[206] Boeckle S, Fahrmeir J, Roedl W, Ogris M, Wagner E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. J Control Release 2006;112(2):240-248.

[207] Bielawski K, Wolczynski S, Bielawska A. Inhibition of DNA topoisomerase I and II, and growth inhibition of MDA-MB-231 human breast cancer cells by bis-

benzimidazole derivatives with alkylating moiety. Pol J Pharmacol 2004;56(3):373-378.

[208] Marschall P, Malik N, Larin Z. Transfer of YACs up to 2.3 Mb intact into human cells with polyethylenimine. Gene Ther 1999;6(9):1634-1637.

[209] Halama A, Kulinski M, Librowski T, Lochynski S. Polymer-based non-viral gene delivery as a concept for the treatment of cancer. Pharmacol Rep 2009;61(6):993-999.

[210] Ohana P, Gofrit O, Ayesh S, Al-Sharef A, Mizrahi A, Birman T, et al. Regulatory sequences of the H19 gene in DNA based therapy of bladder cancer. Gene Ther Mol Biol 2004;8:181-192.

[211] Kleemann E, Jekel N, Dailey LA, Roesler S, Fink L, Weissmann N, et al. Enhanced gene expression and reduced toxicity in mice using polyplexes of low-molecularweight poly(ethyleneimine) for pulmonary gene delivery. J Drug Target 2009;17(8):638-651.

[212] Ogris M, Brunner S, Schüller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 1999;6(4):595-605.

[213] Chollet P, Favrot MC, Hurbin A, Coll JL. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. J Gene Med 2002;4(1):84-91.

[214] Forrest ML, Meister GE, Koerber JT, Pack DW. Partial acetylation of polyethylenimine enhances in vitro gene delivery. Pharm Res 2004;21(2):365-371.

[215] Nishiyama N, Kataoka K. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther 2006;112(3):630-648.

[216] Trivedi R, Kompella UB. Nanomicellar formulations for sustained drug delivery: strategies and underlying principles. Nanomedicine (Lond) 2010;5(3):485-505.

[217] Mallipeddi R, Rohan LC. Progress in antiretroviral drug delivery using nanotechnology. Int J Nanomedicine 2010;5:533-547.

[218] Lin SY, Kawashima Y. Pluronic surfactants affecting diazepam solubility, compatibility, and adsorption from i.v. admixture solutions. J Parenter Sci Technol 1987;41(3):83-87.

[219] Lin SY, Kawashima Y. The influence of three poly(oxyethylene)poly(oxypropylene) surface-active block copolymers on the solubility behavior of indomethacin. Pharm Acta Helv 1985;60(12):339-344.

[220] Yokoyama M, Okano T, Kataoka K. Improved synthesis of adriamycin-conjugated poly(ethylene oxide)-poly(aspartic acid) block copolymer and formation of unimodal micellar structure with controlled amount of physically entrapped adriamycin. J Control Release 1994;32(3):269-277.

[221] Yokoyama M, Fukushima S, Uehara R, Okamoto K, Kataoka K, Sakurai Y, et al. Characterization of physical entrapment and chemical conjugation of adriamycin in polymeric micelles and their design for in vivo delivery to a solid tumor. J Control Release 1998;50(1-3):79-92.

[222] Yokoyama M, Satoh A, Sakurai Y, Okano T, Matsumura Y, Kakizoe T, et al. Incorporation of water-insoluble anticancer drug into polymeric micelles and control of their particle size. J Control Release 1998;1998(55):2-3.

[223] Batrakova EV, Dorodnych TY, Klinskii EY, Kliushnenkova EN, Shemchukova O, Goncharova ON, et al. Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: in vivo evaluation of anti-cancer activity. Br J Cancer 1996;74(10):1545-1552.

[224] Kabanov AV, Nazarova IR, Astafieva IV, Batrakova EV, Alakhov VY, Yaroslavov AA, et al. Micelle formation and solubilization of fluorescent probes in poly(oxyethylene-b-oxypropylene-b-oxyethylene) solutions. Macromol 1995;28(7):2303-2314.

[225] Kabanov AV, Vinogradov SV, Suzdaltseva YG, Alakhov VY. Water-soluble block polycations as carriers for oligonucleotide delivery. Bioconjug Chem 1995;6(6):639-643.

[226] Alakhov VY, Kabanov AV. Block copolymeric biotransport carriers as versatile vehicles for drug delivery. Expert Opin Investig Drugs 1998;7(9):1453-1473.

[227] Torchilin VP, Lukyanov AN, Gao Z, Papahadjopoulos-Sternberg B. Immunomicelles: targeted pharmaceutical carriers for poorly soluble drugs. Proc Natl Acad Sci USA 2003;100(10):6039-6044.

[228] Wang J, Mongayt DA, Lukyanov AN, Levchenko TS, Torchilin VP. Preparation and in vitro synergistic anticancer effect of vitamin k3 and 1,8-diazabicyclo[5,4,0]undec-7-ene in poly(ethylene glycol)-diacyllipid micelles. Int J Pharm 2004;272(1-2):129-135.

[229] Krishnadas A, Rubinstein I, Onyuksel H. Sterically stabilized phopholipid mixed micelles: in vitro evaluation as a novel carrier for water-insoluble drugs. Pharm Res 2003;20(2):297-302.

[230] Ward CM, Read ML, Seymour LW. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. Blood 2001;97(8):2221-2229.

[231] Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev 2001;47(1):113-131.

[232] Mishra D, Kang HC, Bae YH. Reconstitutable charged polymeric (PLGA)2-b-PEI micelles for gene therapeutics delivery. Biomaterials 2011;32(15):3845-3854.

[233] Itaka K, Osada K, Morii K, Kim P, Yun SH, Kataoka K. Polyplex nanomicelle promotes hydrodynamic gene introduction to skeletal muscle. J Control Release 2010;143(1):112-119.

[234] Kataoka K, Harada A, Wakebayashi D, Nagasaki Y. Polyion complex micelles with reactive aldehyde groups on their surface from plasmid DNA and end-functionalized charged block copolymers. Macromol 1999;32:6892-6894.

[235] Strambio-De-Castillia C, Niepel M, Rout MP. The nuclear pore complex: bridging nuclear transport and gene regulation. Nat Rev Mol Cell Biol 2010;11(7):490-501.

[236] Köhler A, Hurt E. Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol 2007;8(10):761-773.

[237] Stewart M. Molecular mechanism of the nuclear protein import cycle. Nat Rev Mol Cell Biol 2007;8(3):195-208.

[238] Wente SR, Rout MP. The nuclear pore complex and nuclear transport. Cold Spring Harb Perspect Biol 2010;2(10):a000562.

[239] Pante N, Aebi U. The nuclear pore complex. J Cell Biol 1993;122(5):977-984.

[240] Mazzanti M, Bustamante JO, Oberleithner H. Electrical dimension of the nuclear envelope. Physiol Rev 2001;81(1):1-19.

[241] Shahin V. Route of glucocorticoid-induced macromolecules across the nuclear envelope as viewed by atomic force microscopy. Pflugers Arch 2006;453(1):1-9.

[242]Kim H, Kim HA, Bae YM, Choi JS, Lee M. Dexamethasone-conjugated polyethylenimine as an efficient gene carrier with an anti-apoptotic effect to cardiomyocytes. J Gene Med 2009;11(6):515-522.

[243] Shahin V, Albermann L, Schillers H, Kastrup L, Schafer C, Ludwig Y, et al. Steroids dilate nuclear pores imaged with atomic force microscopy. J Cell Physiol 2005;202(2):591-601.

[244] Bae YM, Choi H, Lee S, Kang SH, Kim YT, Nam K, et al. Dexamethasone-conjugated low molecular weight polyethylenimine as a nucleus-targeting lipopolymer gene carrier. Bioconjug Chem 2007;18(6):2029-2036.

[245] Mishra V, Gupta U, Jain NK. Biowaiver: an alternative to in vivo pharmacokinetic bioequivalence studies. Pharmazie 2010;65(3):155-161.

[246] Fuchs GS, Mikkelsen S, Knudsen TK, Kappelgaard A-M. Ease of use and acceptability of a new pen device for the administration of growth hormone therapy in pediatric patients: An open-label, uncontrolled usability test. Clin Ther 2009;31(12):2906-2914.

[247] Collier J, Shah R, Gupta A, Sayeed V, Habib M, Khan M. Influence of Formulation and Processing Factors on Stability of Levothyroxine Sodium Pentahydrate. AAPS PharmSciTech 2010;11(2):818-825.

[248] McCluskey SV, Graner KK, Kemp J, Aloumanis V, Ben M, Kupiec T, et al. Stability of fentanyl 5 {micro}g/mL diluted with 0.9% sodium chloride injection and stored in polypropylene syringes. Am J Health Syst Pharm 2009 May 1, 2009;66(9):860-863.

[249] Craig DQ. The mechanisms of drug release from solid dispersions in water-soluble polymers. Int J Pharm 2002;231(2):131-144.

[250] Bulmus V, Chan Y, Nguyen Q, Tran HL. Synthesis and characterization of degradable p(HEMA) microgels: use of acid-labile crosslinkers. Macromol Biosci 2007;7(4):446-455.

[251] Molina MdC, Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators. J Pharm Sci 2001;90(10):1445-1455.

[252] Flores-Fernández GM, Solá RJ, Griebenow K. The relation between moisture-induced aggregation and structural changes in lyophilized insulin. J Pharm Pharmacol 2009;61(11):1555-1561.

[253] Thompson C, Hansford D, Higgins S, Rostron C, Hutcheon G, Munday D. Preparation and evaluation of microspheres prepared from novel polyester-ibuprofen conjugates blended with non-conjugated ibuprofen. J Microencapsul 2009;26(8):676-683.

[254] Miene C, Klenow S, Veeriah S, Richling E, Glei M. Impact of apple polyphenols on GSTT2 gene expression, subsequent protection of DNA and modulation of proliferation using LT97 human colon adenoma cells. Mol Nutr Food Res 2009;53(10):1254-1262.

[255] Holzer M, Vogel V, Mäntele W, Schwartz D, Haase W, Langer K. Physico-chemical characterisation of PLGA nanoparticles after freeze-drying and storage. Eur J Pharm Biopharm 2009;72(2):428-437.

[256] Brautigam K, Bauerschlag DO, Weigel MT, Biernath-Wupping J, Bauknecht T, Arnold N, et al. Combination of enzastaurin and pemetrexed inhibits cell growth and induces apoptosis of chemoresistant ovarian cancer cells regulating extracellular signal-regulated kinase 1/2 phosphorylation. Transl Oncol 2009;2(3):164-173.

[257] Shah N, Kataria H, Kaul SC, Ishii T, Kaur G, Wadhwa R. Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: combinational approach for enhanced differentiation. Cancer Sci 2009;100(9):1740-1747.

[258] Li M, Wang J, Jing J, Hua H, Luo T, Xu L, et al. Synergistic promotion of breast cancer cells death by targeting molecular chaperone GRP78 and heat shock protein 70. J Cell Mol Med 2009;13(11-12):4540-4550.

[259] Thijs L, Richart T, de Leeuw PW, Kuznetsova T, Grodzicki T, Kawecka-Jaszcz K, et al. Morbidity and mortality on combination versus monotherapy: a posthoc analysis of the Systolic Hypertension in Europe trial. J Hypertens 2010;28(4):865-874.

[260] Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, et al. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. J Allergy Clin Immunol 2010;125(2):349-356.e.313.

[261] Fujimoto-Ouchi K, Sekiguchi F, Yamamoto K, Shirane M, Yamashita Y, Mori K. Preclinical study of prolonged administration of trastuzumab as combination therapy after disease progression during trastuzumab monotherapy. Cancer Chemother Pharmacol 2010;66(2):269-276.

[262] Messerli FH, Oparil S, Feng Z. Comparison of efficacy and side effects of combination therapy of angiotensin-converting enzyme inhibitor (benazepril) with calcium antagonist (either nifedipine or amlodipine) versus high-dose calcium antagonist monotherapy for systemic hypertension. Am J Cardiol 2000;86(11):1182-1187.

[263] Johnson MD, MacDougall C, Ostrosky-Zeichner L, Perfect JR, Rex JH. Combination antifungal therapy. Antimicrob Agents Chemother 2004;48(3):693-715.

[264] Schafer C, Shahin V, Albermann L, Hug MJ, Reinhardt J, Schillers H, et al. Aldosterone signaling pathway across the nuclear envelope. Proc Natl Acad Sci USA 2002;99(10):7154-7159.

[265] Shahin V. The nuclear barrier is structurally and functionally highly responsive to glucocorticoids. Bioessays 2006;28(9):935-942.

[266] Kastrup L, Oberleithner H, Ludwig Y, Schafer C, Shahin V. Nuclear envelope barrier leak induced by dexamethasone. J Cell Physiol 2006;206(2):428-434.

CHAPTER 2

RECONSTITUTABLE CHARGED POLYMERIC $(PLGA)_2$-*b*-PEI MICELLES FOR GENE THERAPEUTICS DELIVERY [*]

2.1 Abstract

This study investigated the potential of creating a charged polymeric micelle-based nucleic acid delivery system that could easily be reconstituted by the addition of water. $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ (PLGA MW 36 kDa, bPEI M_w 25 kDa, PLGA:bPEI block ratio = 2) was synthesized and used to prepare cationic micelles. The copolymer retained proton-buffering capability from the bPEI block within the endosomal pH range. Micelle/pDNA complexes retained their particle size (100-150 nm) and zeta potential (30-40 mV) following reconstitution. It was found that adding a small amount of low molecular weight bPEI (1.8 kDa) completely shielded pDNA in the micelle/pDNA complexes and enhanced transfection efficiency 50-100 fold for both fresh and reconstituted complexes without affecting complex size. Transfection efficiency for "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes was 16-fold higher than its "fresh" counterpart. Although transfection levels achieved using "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ complexes were 3.6-fold lower than control "fresh" $bPEI_{25kDa}$/pDNA (N/P 5) complexes, transfection levels were 39-fold higher than

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"reconstituted" $bPEI_{25kDa}$/pDNA (N/P 5) complexes. The micelle/pDNA/$bPEI_{1.8kDa}$ system showed very low cytotoxicity in MCF7 cells even with pDNA doses up to 20 μg, and transfection levels increased linearly with increasing pDNA dose. These results indicate that this PLGA-*b*-bPEI polymeric micelle-based system is well suited as a reconstitutable gene delivery system, and has high potential for use as a delivery system for gene therapy applications.

2.2. Introduction

Gene therapy is a technique that uses genes to treat or prevent disease. There are several applications for gene therapy including replacing a mutated gene with a healthy copy of gene [1, 2], introducing a new gene to help fight disease [3, 4], or inactivating a mutated gene that is not functioning correctly [5]. This technique has the potential for broad scale application and impact in treating both acquired and inherited diseases. Although there has been some success using viral vector systems as vehicles for gene transfection *in vitro* and *in vivo*, the large number of drawbacks associated with viral-based carrier systems has increased interest in the development of nonviral delivery systems [6]. Concerns about the integration of delivered exogeneous DNA into the host genome [7-9], cytotoxicity [10], tumorigenicity [11], and immunogenicity [12] have increased interest in finding alternative delivery systems [6, 13].

Nonviral based delivery systems are typically classified into two main categories – lipids and polymers. With attractive biological traits such as low immunogenicity, no tumorigenicity, and excellent biocompatibility, polymer-based systems offer the ability to engineer carrier systems with customized features that can be adapted to suit any

system [6]. Functionalities include attaching targeting moieties to the nonviral polymer carrier for targeting specific cell types [14-16], adding endosomolytic agents to disrupt the endosome [17, 18], increasing nuclear delivery by targeting the cell nucleus [19, 20], or designing degradable polymers to decrease toxicity [21-23].

Many different combinations of polymeric/oligomeric blocks in single polymer systems have been created and studied in an effort to increase utility [24]. Block copolymer micelle systems in particular have gained interest in recent years as promising drug delivery vehicles because of their ability to prolong circulation in the blood and their ability to modulate the pharmacokinetics of drugs [25]. For gene delivery, micelle copolymers having a hydrophobic block-charged hydrophilic block architecture usually assemble into a core-shell micelle structure, creating the potential to load hydrophobic drugs and gene drugs into the different compartments (core and shell), respectively. Some recent examples of such copolymers include poly(ε-caprolactone)-bPEI$_{1.8kDa}$ [26], poly((*N*-methyldietheneamine sebacate)-*co*-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide] sebacate) [27], and poly(dimethylaminoethyl methacrylate)-poly(ε-caprolactone)-poly(dimethylaminoethyl methacrylate) [28].

Even so, nonviral systems are still a long way from being readily available as a mainstream therapeutic option. Some important formulation considerations for clinical usage are drug concentration [29], ease of administration [30], and formulation stability during storage [31, 32]. Many formulations made from liposomes or polymer-based systems exist as colloidal liquids, which are generally stable for short periods of time

[33]. However, some polymer systems are designed to be gradually degradable by hydrolysis so long-term storage in liquid form is not a viable option for these types of systems [34]. In addition, many formulations can become unstable if kept in liquid form during long-term storage due to a variety of issues including degradation of the nonviral vector and/or drug [35], formation of insoluble aggregates [36], unwanted drug release [37], and loss of bioactivity [38]. One method to overcome the stability limitations of storing liquid formulations is to lyophilize the formulation, allowing storage in a powder form. When the formulation is required, it can be reconstituted though the simple addition of water or buffer. While some success has been seen using this method, many groups have additionally added cryoprotective agents [39, 40] or modified the formulation in some way such as through PEGylation [41, 42] or physical cross-linking [43]. Unfortunately these formulations showed some changes in their characteristics following reconstitution such as increases in particle size [39]. The ideal scenario would be to have a formulation that can be lyophilized, be reconstituted easily by simply adding water or buffer, and most importantly retain its original chemical, physical and biological properties.

This study was designed to investigate the possibility of creating a charged polymeric micelle-based gene therapeutic delivery system that maintains efficacy following lyophilization and reconstitution. Branched polyethyleneimine (bPEI) and poly(lactide-*co*-glycolide) (PLGA) were selected to create our micelle system. The hydrophilic cationic component $bPEI_{25kDa}$ (M_w 25 kDa) has been used as the gold standard for polymeric vectors but its viscosity and sticky characteristics have limited its

use in a dried state. The hydrophobic degradable component PLGA has been used in dried nano/microparticles form but its water-insolubility limits its use for creating formulations containing biological therapeutics in aqueous buffers. In order to extract the favorable features from both $bPEI_{25kDa}$ and $PLGA_{36kDa}$, these two polymers were chemically linked together and the resulting block copolymer was used to create charged micelles which could interact with biological agents in aqueous solution and could also be stored as a dried formulation. Physicochemical and biological comparisons between freshly-prepared micelle-based gene complexes and reconstituted micelle-based gene complexes were evaluated in terms of particle size, zeta potential, pDNA condensation ability, and cell transfection efficiency.

2.3. Materials and Methods

2.3.1. Materials

Poly(lactide-*co*-glycolide) ($PLGA_{36kDa}$; Resomer®503H; lactide:glycolide = 1:1 (mole/mole); approximate MW 36 kDa) having a carboxylic group at one end was purchased from Boehringer Ingelheim Pharm GmbH & Co. KG (Germany). Two bPEIs having M_r 1.8 kDa ($bPEI_{1.8kDa}$) and M_w 25 kDa ($bPEI_{25kDa}$; M_n 10 kDa) were purchased from Polysciences, Inc. (Warrington, PA) and Sigma-Aldrich Co. (St. Louis, MO) respectively. Triethylamine (TEA), *N*,*N*'-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), RPMI1640 cell culture medium powder, sodium bicarbonate, $_D$-glucose, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), human recombinant insulin, Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT),

dimethyl sulfoxide (DMSO), and agarose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS), penicillin/streptomycin, and 0.25% trypsin/EDTA were purchased from Gibco BRL (Grand Island, NY). YOYO-1 dye was purchased from Invitrogen, Inc (Carslbard, CA). Ethidium bromide (EtBr) and the Biocinchoninic Acid Protein Assay Kit (BCA) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Luciferase assay kit was obtained from Promega Co. (Madison, WI). Spectrapor dialysis membrane MWCO 15 kDa was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Plasmid DNA (pDNA) encoded with the firefly luciferase reporter gene (gWiz-Luc) was purchased from Aldevron, Inc. (Fargo, ND).

2.3.2. Cell Culture

In this study, MCF7 cells (human breast adenocarcinoma cell line) were used for determining the cytotoxicity of micelles and polyplexes and for polyplex transfection. All experiments used MCF7 cells cultured in RPMI1640 cell culture medium supplemented with insulin (4 mg/L), glucose (2 g/L), 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics at 37 °C in humidified air containing 5% CO_2.

2.3.3. Synthesis and Characterization of Amphiphilic Block Copolymers

A block copolymer composed of $PLGA_{36kDa}$ and $bPEI_{25kDa}$ ($(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$) was synthesized by conventional condensation between the carboxylic acid group from $PLGA_{36kDa}$ and the amine groups from $bPEI_{25kDa}$ as shown in Fig. 2.1(a). In detail, $PLGA_{36kDa}$ (100 µmol) and $bPEI_{25kDa}$ (50 µmol) were dissolved separately in DMSO (100 mL) and stirred independently for 4 hours. Then the two polymer solutions were mixed

together along with DCC (1 mmol), NHS (1 mmol), and TEA (0.1 mL) and polymerization was carried out at room temperature (RT) for 48 hours under constant stirring. About 100 mL of deionized (DI) water was added to the reaction mixture and stirred for 2 hours; then the polymer solution was transferred to a dialysis tube (MWCO 15 kDa) and dialyzed against DI water for two days to remove any unreacted $bPEI_{25kDa}$. Unreacted $PLGA_{36kDa}$ was removed by filtration at a later stage following micelle formation. The dialysis solution was lyophilized to obtain the resulting block copolymer. The chemical structure was confirmed by ^{1}H-NMR spectroscopy.

2.3.4. Preparation and Characterization of Micelles

Micelles were prepared using standard dialysis techniques. As shown in Fig. 2.1(b), $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ was dissolved in DMSO at a concentration of 10 mg/mL and stirred for 4 hours at RT. HEPES buffer (20 mM, pH 7.4) was added to the reaction mixture at an equivalent volume to DMSO and stirred for an additional 2 hours. The reaction mixture was transferred to a dialysis membrane (MWCO 15 kDa) and dialyzed against DI water for 24 hours. The resulting micelle solution was filtered using a 0.22 μm filter to remove any unreacted $PLGA_{36kDa}$ remaining from the synthesis (which precipitates in water) and the remaining solution was lyophilized and stored at -20°C until needed.

Particle size and zeta potential of freshly-prepared micelles and reconstituted micelles were measured in HEPES 20mM buffer at RT using a Zetasizer $3000HS_A$ (Malvern Instruments Inc., Worcestershire, UK) with a fixed wavelength of 677 nm and a constant angle of 90°.

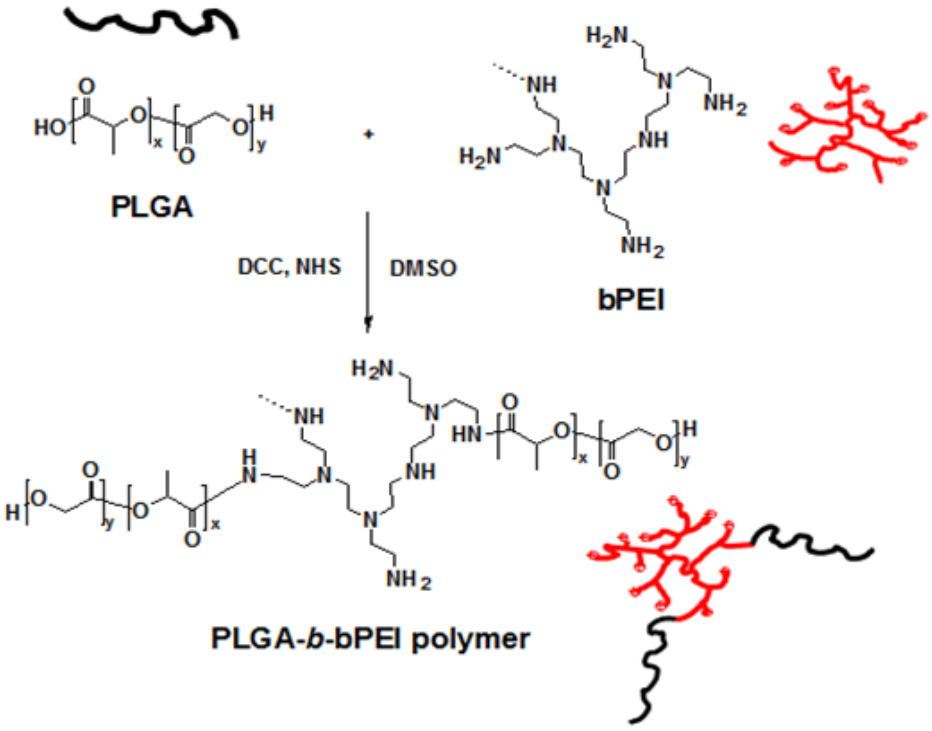

Figure 2.1: Synthesis of PLGA-*b*-bPEI copolymer and schematic of micelle preparation and complex formation (a) reaction schematic for $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ copolymer

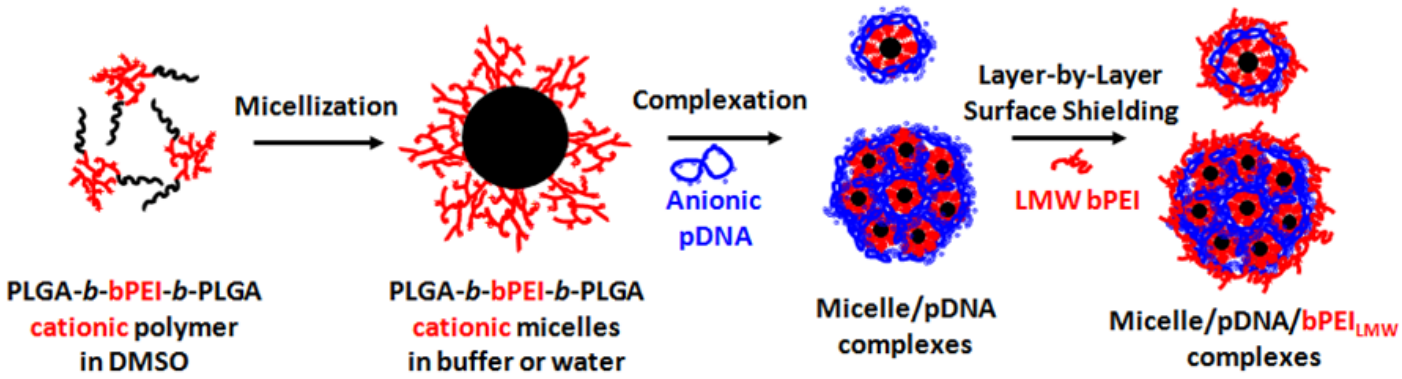

Figure 2.1 continued: (b) schematic representation of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles, micelle/pDNA complexes, and micelle/pDNA/$bPEI_{LMW}$ complexes.

To evaluate whether the $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles maintained any proton buffering capacity from the $bPEI_{25kDa}$ block, micelles were titrated using traditional acid-base titration methods [18, 23]. $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles were dispersed in a fixed volume of 150 mM NaCl and titrated from pH 7.4 to pH 3 using 0.1 N HCl. The proton buffering ability of the $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles was compared to $bPEI_{25kDa}$ alone within the pH range 7.4-5.1 because this pH range correlates with typical endolysosomal pHs. Buffering capacity (%) of the polymers was calculated using the following equation [44]:

$$\text{Buffering capacity } (\%) = \frac{\Delta V_{HCl} \times C_{HCl}}{N} \times 100(\%)$$

where ΔV_{HCl} is the volume of the HCl solution (0.1 M) which decreased the pH value of the polymer solution from pH 7.4 to pH 5.1, C_{HCl} is the concentration of the HCl solution (0.1 M), and N is the total moles of protonable amine groups contained in the polymer.

Cytotoxicity of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles was assessed using standard protocols for MTT-based cell viability assay [18, 23]. MCF7 breast cancer cells were seeded in a 96-well plate at a density of 5000 cells/well in culture medium (0.1 mL). Sample solutions (10 µL) at various concentrations were added to each well and incubated for 24 hours. MTT solution (10 µL, 5 mg/mL) was added to each well and the plates were incubated for 4 hours at 37°C in a cell culture incubator. Media was completely removed, 100 µL of DMSO was added to each well to dissolve the formazan metabolites, and the plate was incubated for 10 minutes at 37°C. Absorbance was

measured at 570 nm using a microplate reader (SpectraMax® M2, Molecular Devices, Sunnyvale, CA) and used to calculate cell viability.

2.3.5. Preparation and Characterization of Polyplexes

Micelle solutions prepared by standard dialysis techniques and used immediately are referred to as “fresh” micelles. “Fresh” micelles that were lyophilized and then reconstituted by adding DI water are referred to as “reconstituted” micelles. Micelle solutions (fresh or reconstituted) were mixed with pDNA in HEPES buffer (20 mM, pH 7.4) containing 5% glucose (HBG) and incubated at RT for 30 minutes to form polyplexes. Some polyplexes were additionally mixed with a small amount of $bPEI_{1.8kDa}$ following complexation at a weight ratio of 2.5µg $bPEI_{1.8kDa}$ per 1µg pDNA. This method was used to create polyplexes for particle size and zeta potential measurements, gel retardation studies, gene transfection and cell viability tests.

Complexation between micelles and pDNA was evaluated by gel retardation and dye quenching methods [16, 18]. For the gel retardation studies, polyplex solution (0.5 µg pDNA in 10 µL) was loaded onto a 0.8 % agarose gel containing ethidium bromide (EtBr) (100 ng/mL). Electrophoresis was run using 0.5X Tris-buffer containing boric acid and EDTA (TBE) at 100 V for 60 minutes. The gel was imaged using an Alpha Innotech FluorChem FC2® instrument with Alpha View Software (Cell Biosciences, Santa Clara CA).

For the dye quenching assays, pDNA was mixed with EtBr at a mole ratio of five nucleotides per one EtBr and pre-incubated in the dark at RT for 30 minutes. Using EtBr-intercalated pDNA, polyplexes were prepared following the same method described above. Polyplexes were excited at 515 nm and the emitted fluorescence was measured

at 595 nm using a Spectramax® M2 spectraphotometer (Molecular Devices, Sunnyvale CA).

Particle size and zeta potential of fresh polyplexes and reconstituted polyplexes were measured at RT using a Zetasizer 3000HS_A (Malvern Instruments Inc., Worcestershire, UK) with a fixed wavelength of 677 nm and a constant angle of 90°.

In vitro transfection of fresh polyplexes and reconstituted polyplexes was evaluated in MCF7 cells using previously reported methods [45]. The cells were seeded at a density of 5×10^5 cells/well in 6-well plates and cultured for 24 hours in culture medium (2 mL). One hour prior to transfection, the cell culture medium was removed and replaced with serum-free insulin-free culture medium (2 mL). Polyplexes were added to the wells (20 µL volume, pDNA content was fixed at 1 µg/well) and cells were incubated for 4 hours at 37°C. The medium was replaced with the complete culture medium (supplemented with serum and insulin) and incubated for another 44 hours. Following incubation, culture medium was removed and cells were rinsed once with DPBS and lysed using a reporter lysis buffer. Luciferase gene expression (relative luminescence units (RLU)) was quantified by following the manufacturer's protocol for the luciferase assay. Protein content in the cells was evaluated by the BCA™ protein assay. Gene transfection efficiency is reported as RLU/mg protein.

In vitro cytotoxicity of fresh polyplexes and reconstituted polyplexes with or without $bPEI_{1.8kDa}$ was monitored by MTT-based cell viability assay. The experimental procedure was the same as previously described for *in vitro* transfection except the cell number used (2.5×10^5 cells/well; 12-well plates) and the polyplex dose (10 µL; 0.5 µg

pDNA/well). After completing the 48-hour transfection procedure, MTT solution (0.1 mL; 5 mg/mL) was added to the cells (in 1 mL of culture medium). After an additional 4-hour incubation, the MTT-containing medium was removed. The resulting formazan crystals produced by living cells were dissolved in 1 mL of DMSO and absorbance was measured at 570 nm using a microplate reader.

For the pDNA dose-dependent transfection and cytotoxicity studies, the final glucose concentrations of transfection media and culture media with polyplex solutions were adjusted to 2 g/L to avoid any potential glucose-induced effects on cell viability/toxicity.

Cellular uptake of the polyplexes was monitored using flow cytometry following methods described elsewhere [23]. Briefly, polyplexes were prepared using pDNA stained with YOYO-1 dye and added to cells preseeded in 6-well culture plates. After incubation for 4 hours, cells were detached and fixed with 4% paraformaldehyde solution. Flow cytometry (FACScan Analyzer, Becton-Dickinson, Franklin Lakes, NJ) was used to monitor cells containing fluorescence and a primary argon laser (488nm) and fluorescence detector (530 ± 15nm) were used to detect the presence of YOYO-1 dye. The number of YOYO-1 stained-pDNA molecules contained per cell was counted and reported per event.

The statistical significance of the data was evaluated by conducting unpaired Student's t-test with a confidence level of $p<0.05$, one-variable analysis of variance (ANOVA) and two-variable analysis of variance (ANOVA).

2.4. Results and Discussion

2.4.1. Synthesis and Characterization of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ Block Polymers

Cationic amphiphilic block copolymers composed of $PLGA_{36kDa}$ and $bPEI_{25kDa}$ were synthesized by a conjugation reaction between monocarboxylated $PLGA_{36kDa}$ and the primary amines of $bPEI_{25kDa}$ (Fig. 2.1(a)). Copolymer synthesis and block ratio ($PLGA_{36kDa}$ to $bPEI_{25kDa}$) were confirmed by ^{1}H-NMR. In d_6-DMSO, $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ polymer was confirmed by observing peaks centered around δ=5.20 for C(=O)-C**H**$_2$-O of glycolide, δ=1.45 for C**H**$_3$- of lactide, δ=4.85 for C(=O)-C**H**(CH_3)-O of lactide, and δ= 3.35, 3.80, and 4.10 for the -C**H**$_2$- groups neighboring the primary, secondary, tertiary amines of $bPEI_{25kDa}$ (Fig. 2.2)**.** However, the -C**H**$_2$- groups of $bPEI_{25kDa}$ showed broad absorption due to the overlapping absorption of the amine groups of $bPEI_{25kDa}$ [46] as seen in Fig. 2.2. In addition, the ratio of lactide and glycolide in $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ from the spectra taken in DMSO did not match the original 1:1 mole ratio contained in the $PLGA_{36kDa}$. This could be due to the weakly soluble character of $bPEI_{25kDa}$ in DMSO; some fraction of the lactide and glycolide could have been hidden from detection by the $bPEI_{25kDa}$ domain.

Thus, to obtain accurate block ratios for $(PLGA_{36kDa})_2$-b-$bPEI_{25kDa}$, the $PLGA_{36kDa}$ block was completely degraded into its glycolic acid and lactic acid units by base-catalyzed hydrolysis in D_2O containing 1 N NaOH. The degradation products of $(PLGA_{36kDa})_2$-b-$bPEI_{25kDa}$ were very soluble in the aqueous solution. Based on the integration ratio of glycolic acid (or lactic acid) and $bPEI_{25kDa}$ (Fig. 2.3), the block ratio of $PLGA_{36kDa}$ per $bPEI_{25kDa}$ was 1.93 and the estimated molecular weight (based on ^{1}H-NMR

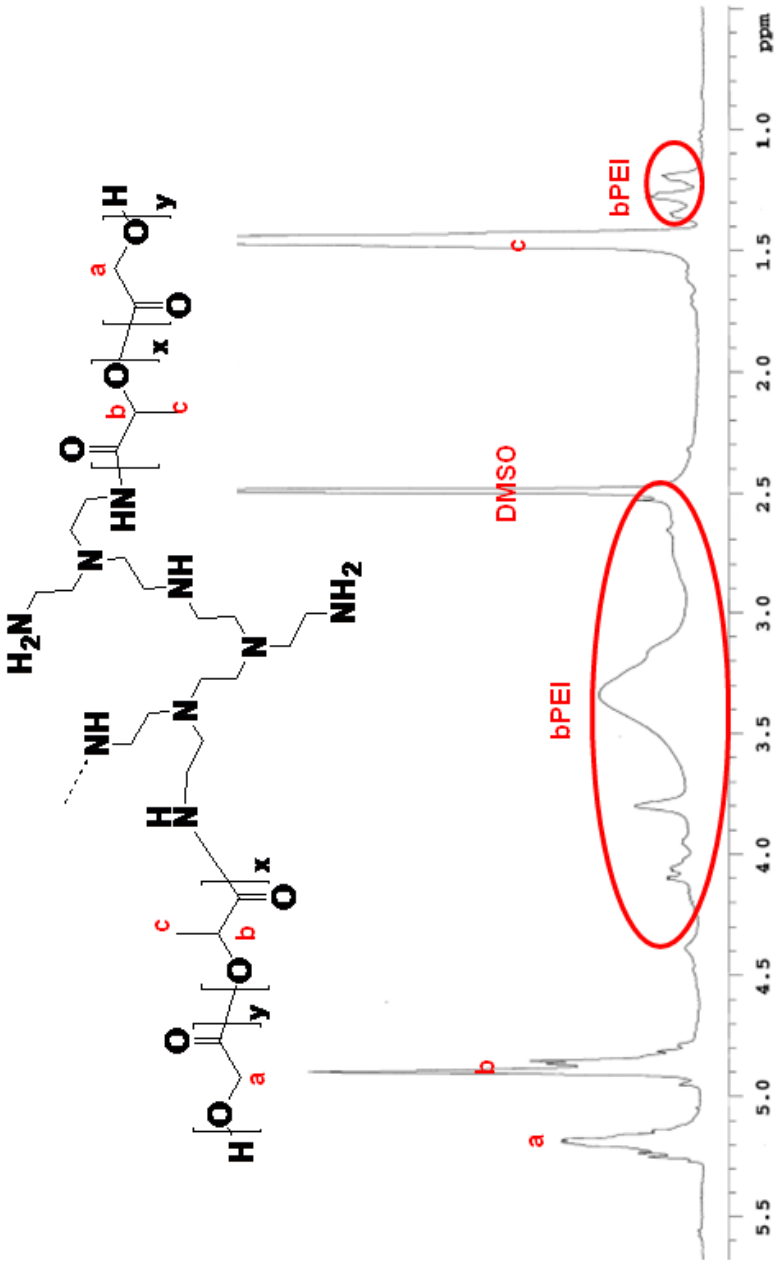

Figure 2.2: ^{1}H-NMR spectra of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ in d_6-DMSO.

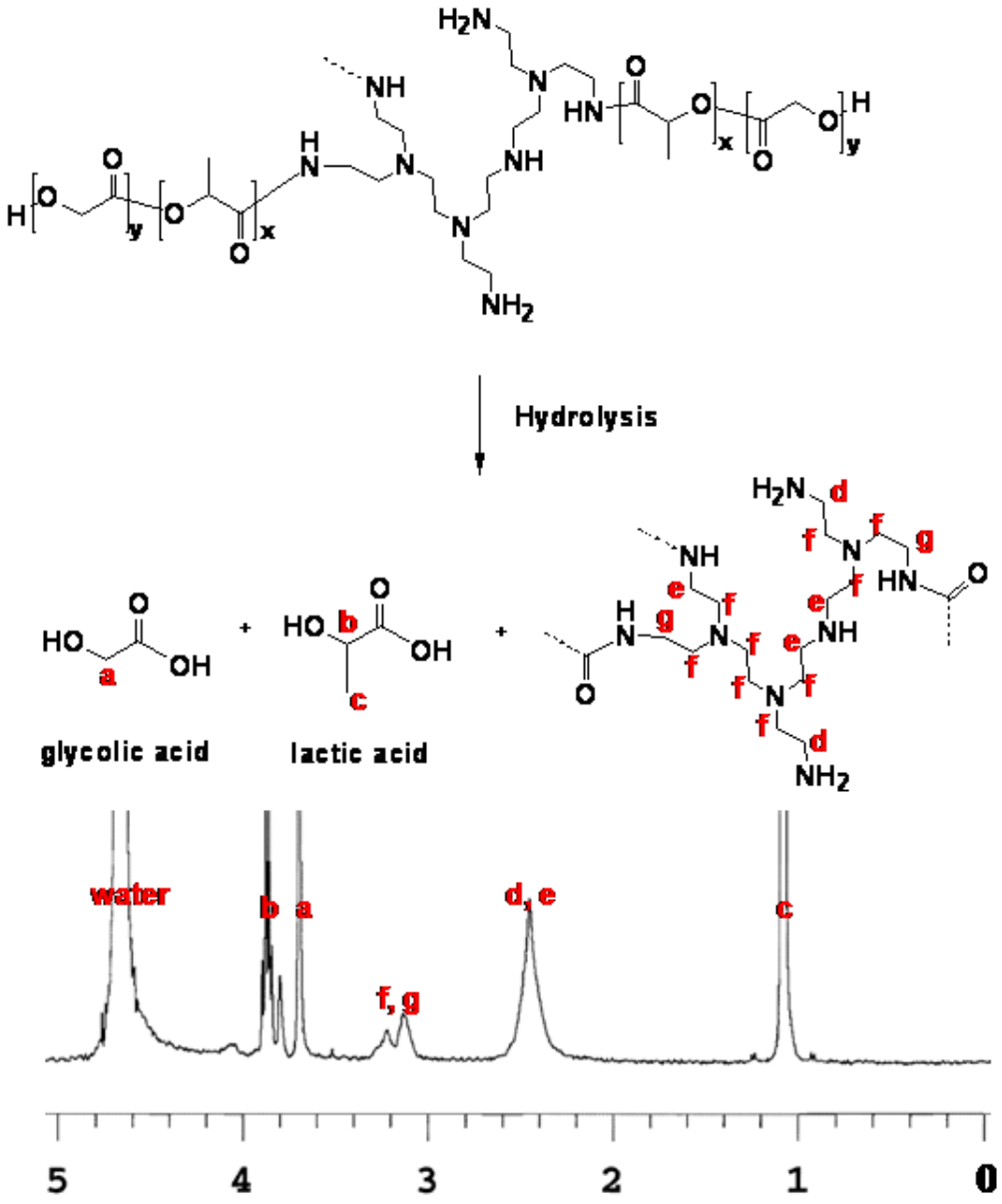

Figure 2.3: ^{1}H-NMR spectra of hydrolyzed $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ in 1 N NaOH-containing D_2O.

data) was approximately 79.5 kDa based on Mn (~94.5 kDa based on Mw). Therefore, as designed, the block copolymer is very close to a triblock copolymer of $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$-*b*-$PLGA_{36kDa}$ ($(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$).

2.4.2. Preparation and Characteristics of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ Micelles

Utilizing the amphiphilic character of the block copolymer, positively-charged polymeric micelles were formed using conventional dialysis methods (Fig. 2.1(b)). The micelles formed a core-shell structure where $PLGA_{36kDa}$ formed the core and $bPEI_{25kDa}$ formed the corona (or shell). Cationic micelles made from $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ had an average diameter of 50-60 nm (Z_{avg}-particle size relevant to hydrodynamic size) and zeta potential of 15-25 mV (Fig. 2.4). Cationic micelles in aqueous solution maintained their size and zeta potential because the strong positively-charged corona prevented the formation of aggregated micelles due to repulsion. The micelle core made from relatively high molecular weight PLGA (MW 36 kDa) can be characterized by strong hydrophobicity [47-49] and slow degradation rate [47, 50, 51].

Although the aqueous colloidal stability of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles can be assured for up to 4 days when stored at 4°C, long-term exposure to various storage conditions (humidity, temperature, aqueous solution, etc.) may alter the physicochemical characteristics (particle size and zeta potential) of the micelles and could damage the biological function of any biotherapeutic agents loaded into the micelles. Storage in a powder form could alleviate the potential pitfalls associated with storage in solution form. Thus, particle size and zeta potential of the micelles were

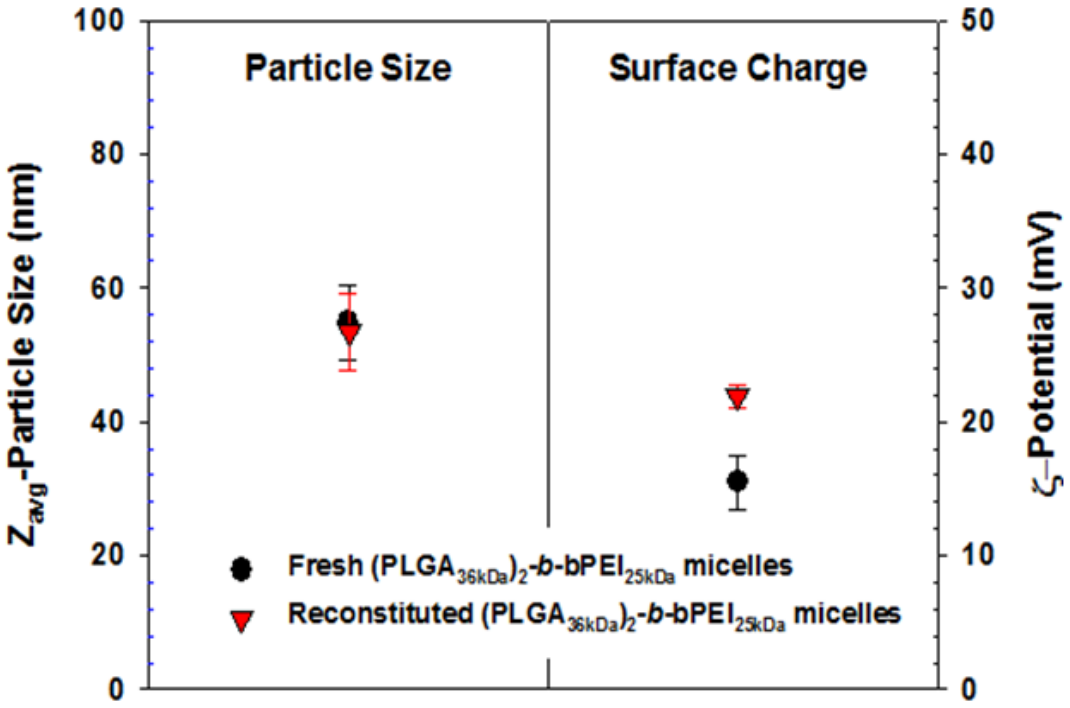

Figure 2.4: Particle size and zeta potential of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles before and after reconstitution. (n=5; mean ± SD)

monitored to evaluate whether the colloidal stability of the micelles was preserved after lyophilization. As shown in Fig. 2.4, particle size and zeta potential of reconstituted micelles were similar to those of fresh micelles.

It is well known that $bPEI_{25kDa}$ has very strong proton-buffering capabilities. To determine how much proton-buffering capacity was preserved in the $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles, $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ (5 mg/mL; 43.88 µmol of amines in 3 mL) was dissolved in 150 mM NaCl and titrated from pH 7.4 to pH 3.0 by adding 0.1 N HCl. The buffering capacity of the copolymer was compared to $bPEI_{25kDa}$ at 0.5 mg/mL (34.88 µmol of amines in 3 mL) and 0.75 mg/mL (52.33 µmol of amines in 3 mL) because the weight percent of $bPEI_{25kDa}$ in the copolymer was approximately 12.6% (based on M_n). As shown in Fig. 2.5, the $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ copolymer retained broad proton buffering ability within the endosomal pH range (specifically pH 5.1 to pH 7.4) similar to $bPEI_{25kDa}$, although the buffering capacity (10.6%) was lower after copolymerization as compared to free $bPEI_{25kDa}$ (average 17.2% from two different concentrations). The reason for this decrease is most likely because there is decreased accessibility of protons to protonate secondary or tertiary amines located near the linkage to the strong hydrophobic PLGA block which prevents them from becoming ionized as effectively following polymerization, thus reducing the overall buffering capability.

The toxicity of the polymeric micelles was compared to the toxicity of control polymers (*i.e.*, $bPEI_{25kDa}$ and $bPEI_{1.8kDa}$) as shown in Fig. 2.6. As expected, the cytotoxicity of the micelles was quite low compared to $bPEI_{25kDa}$ alone. The IC_{50} of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ was approximately 110 µg/mL, much higher than $bPEI_{25kDa}$ (~ 12 µg/mL).

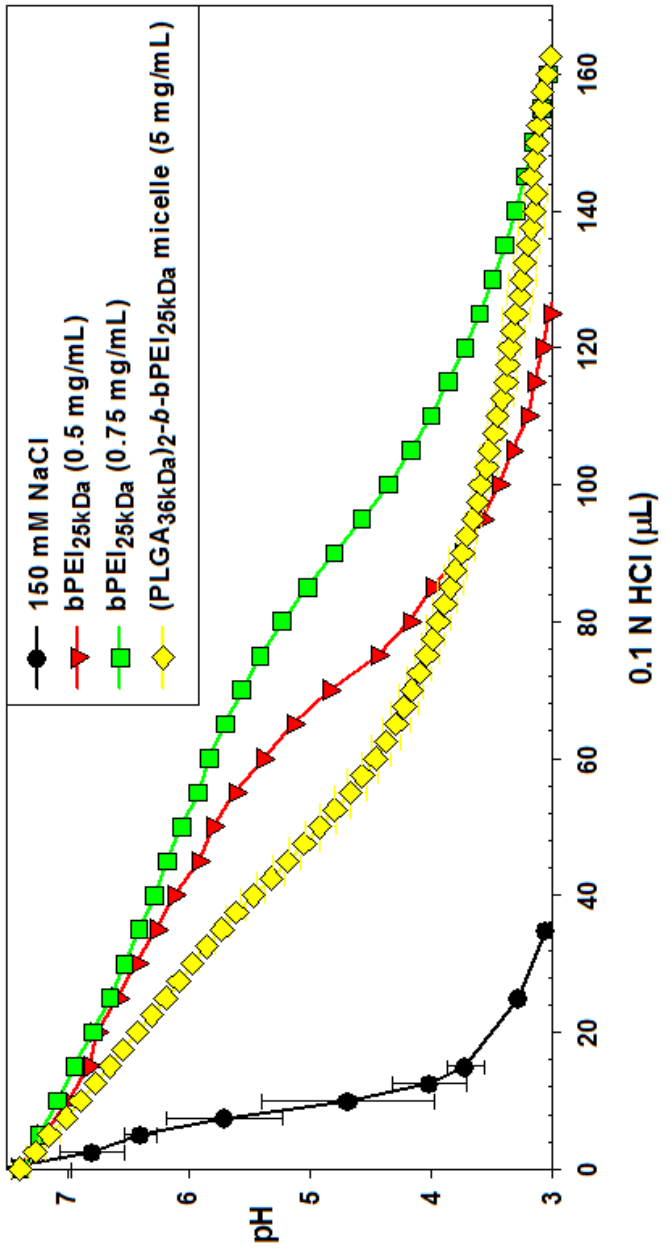

Figure 2.5: Proton buffering capacity of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles and $bPEI_{25kDa}$. (n=3; mean ± SD)

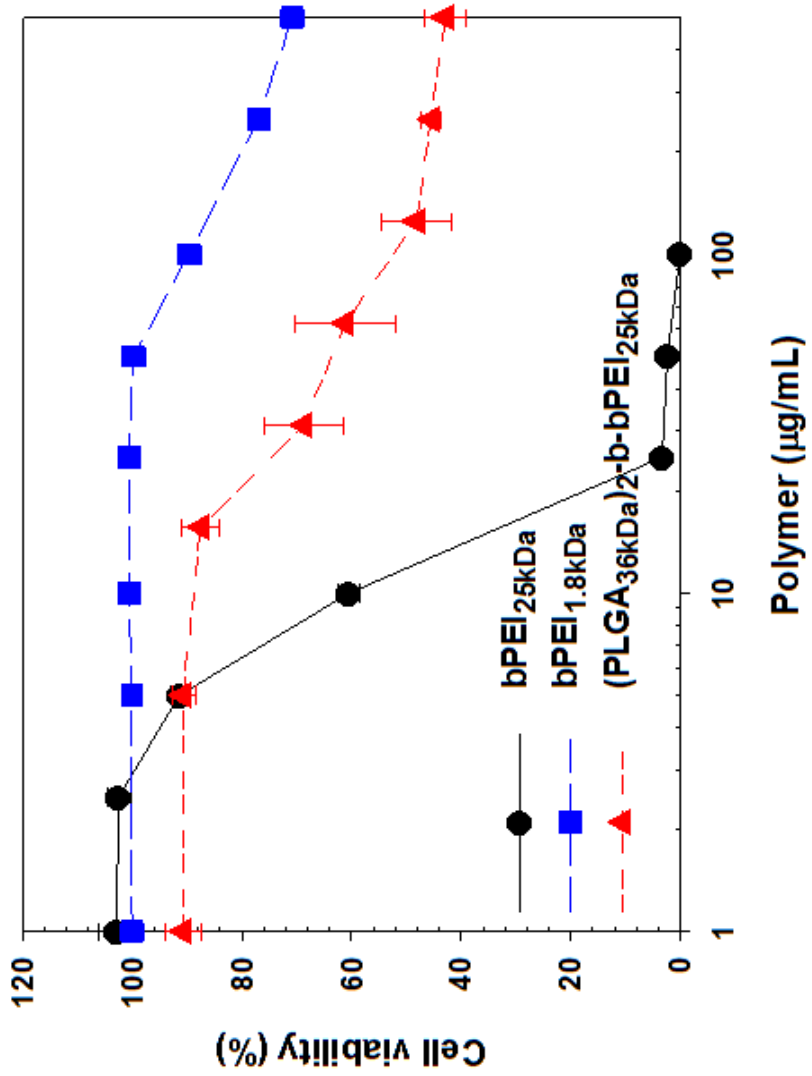

Figure 2.6: Cytotoxicity of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles, $bPEI_{25kDa}$, and $bPEI_{1.8kDa}$ in MCF7 cells. (n=6; mean ± SD)

However, if the weight fraction of $bPEI_{25kDa}$ contained in the copolymer is considered, then the IC_{50} of PEI_{25kDa} in $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ (13.9 μg/mL) is not significantly different from $bPEI_{25kDa}$ alone. In addition, the cytotoxicity contribution from $bPEI_{1.8kDa}$ was negligible within the concentration range used in the transfection studies (2.5 μg per 1 μg pDNA; 1.25-25 μg/mL $bPEI_{1.8kDa}$ for 1-20 μg pDNA), with cell viabilities higher than 90% for the concentrations of $bPEI_{1.8kDa}$ used.

2.4.3. Preparation and Characteristics of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ Micelle-Based Polyplexes

Reconstituted $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles were complexed with pDNA and characterized to further address whether $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelle/pDNA complexes (abbreviated as micelle/pDNA complexes) could be used to produce exogenous proteins in cells via transfection of exogenous therapeutic pDNA. To evaluate this potential, the first step was to form electrostatic complexes between cationic micelles and anionic pDNA. Although most pDNA appeared to complex with the micelles at low weight ratios (WR) of micelle to pDNA (*e.g.*, WR <3), some pDNA remained uncomplexed (Fig. 2.7). With higher WRs (*e.g.*, WR 25), all of the pDNA complexed with the micelles but some pDNA still appeared to be exposed or only weakly complexed (Fig. 2.8). Nevertheless, micelle/pDNA complexes having WR ≥ 15 were approximately 50-120 nm in diameter and had a strong positive zeta potential (20-25 mV) (Fig. 2.9). When micelle/pDNA complexes with WR 15-30 were used to transfect MCF7 cells, the micelle/pDNA complexes showed approximately 10^3-10^4 lower transfection efficiency

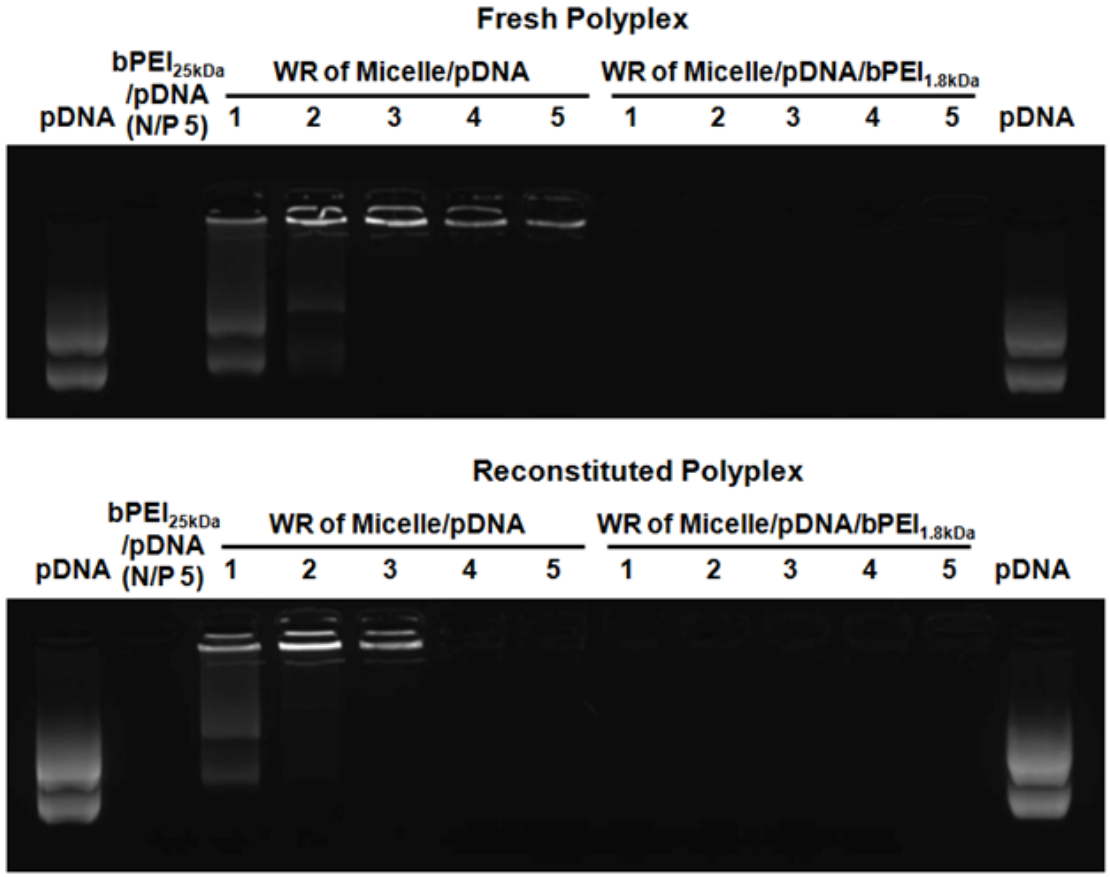

Figure 2.7: Surface shielding effect of $bPEI_{1.8kDa}$ on gene condensation of micelle/pDNA complexes (a) using agarose gel retardation (n=3; mean ± SD)

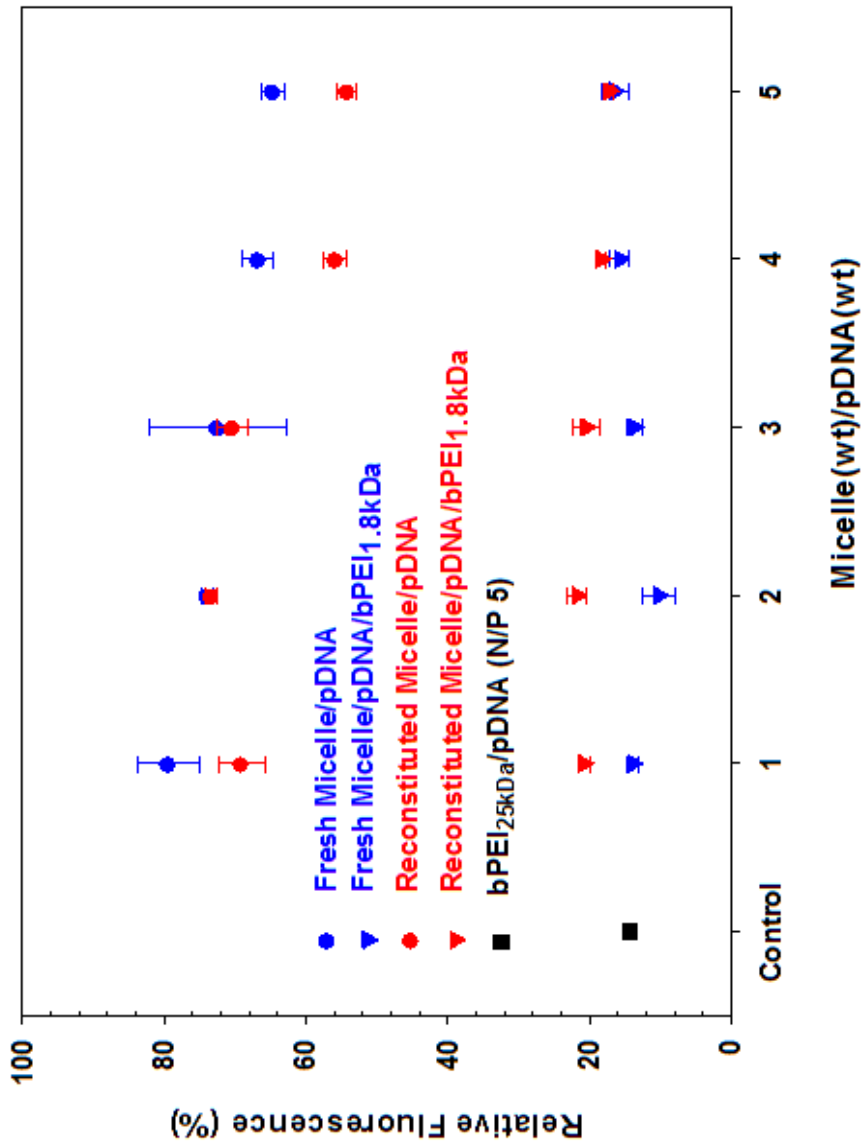

Figure 2.7 continued: (b) using dye quenching assay (n=3; mean ± SD)

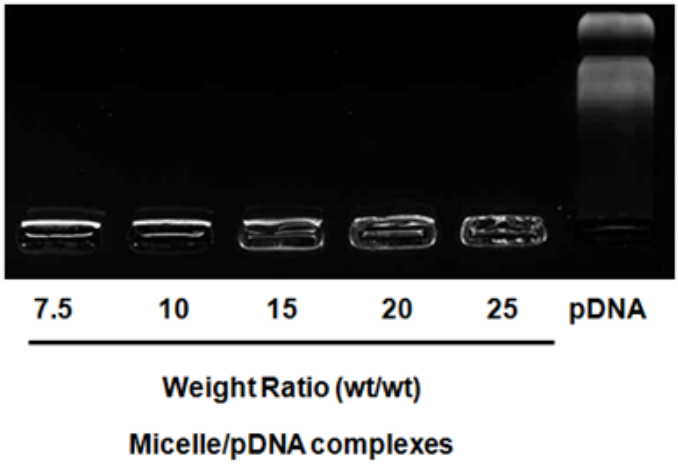

Figure 2.8: Condensation of pDNA with $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles at high weight ratios. (n = 3; mean ± SD)

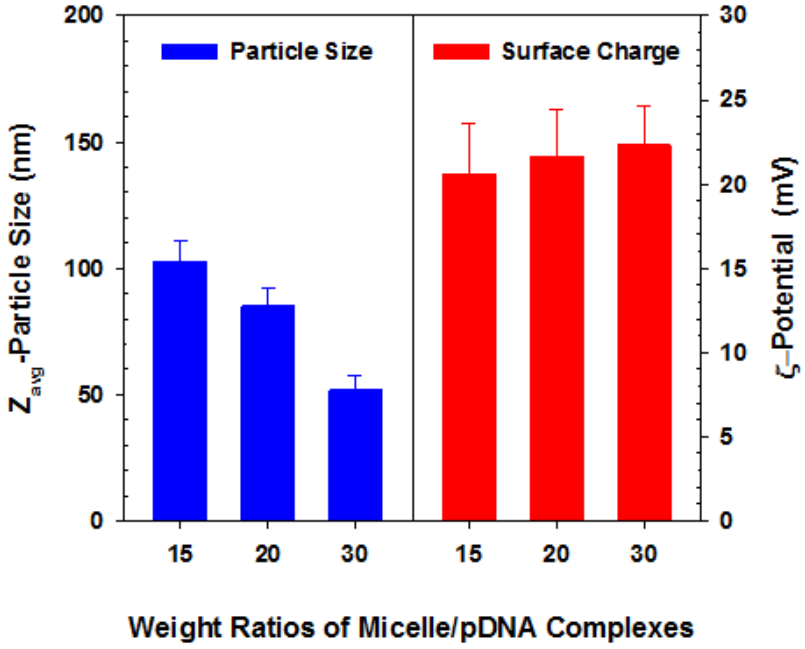

Figure 2.9: Particle size and zeta potential of micelle/pDNA complexes at high weight ratios. (n = 3; mean ± SD)

than $bPEI_{25kDa}$/pDNA complexes (N/P 5) (Fig. 2.10). This result may be due to either weak complexation between pDNA and the micelles or the presence of partially exposed pDNA on the surface of the micelle/pDNA complexes.

To solve the aforementioned possible issues with micelle/pDNA complexes, low molecular weight (LMW) bPEIs were introduced into the micelle/pDNA complexes. LMW bPEIs could shield any exposed pDNA on the surface of the complexes through electrostatic layer-by-layer (LBL) attraction. In addition, LMW bPEIs could fill up any free volume between the cationic micelles and pDNAs and induce tighter complexation. From among the commercially available bPEIs, the lowest MW bPEI (*i.e.*, $bPEI_{0.8kDa}$) was selected as the first candidate to prove these hypotheses. However, micelle/pDNA complexes shielded with $bPEI_{0.8kDa}$ (micelle/pDNA/$bPEI_{0.8kDa}$ complexes) still had some exposed pDNA, as illustrated by the detection of EtBr-intercalated pDNA from those complexes in the gel retardation data (Fig. 2.11). The next smallest available bPEI ($bPEI_{1.8kDa}$) was selected next to see if it could endow stronger interaction between the LMW bPEIs and pDNA. Introducing $bPEI_{1.8kDa}$ resulted in no detection of EtBr-intercalated pDNA in "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complexes (Fig. 2.7(a)). In addition, dye quenching results clearly indicate that $bPEI_{1.8kDa}$ completely prevented pDNA exposure on polyplex surface (Fig. 2.7(b)). These findings indicate that $bPEI_{1.8kDa}$ completely shielded any exposed pDNA on the surface of "fresh" micelle/pDNA complexes or that $bPEI_{1.8kDa}$ strengthened the complexation between micelles and pDNA.

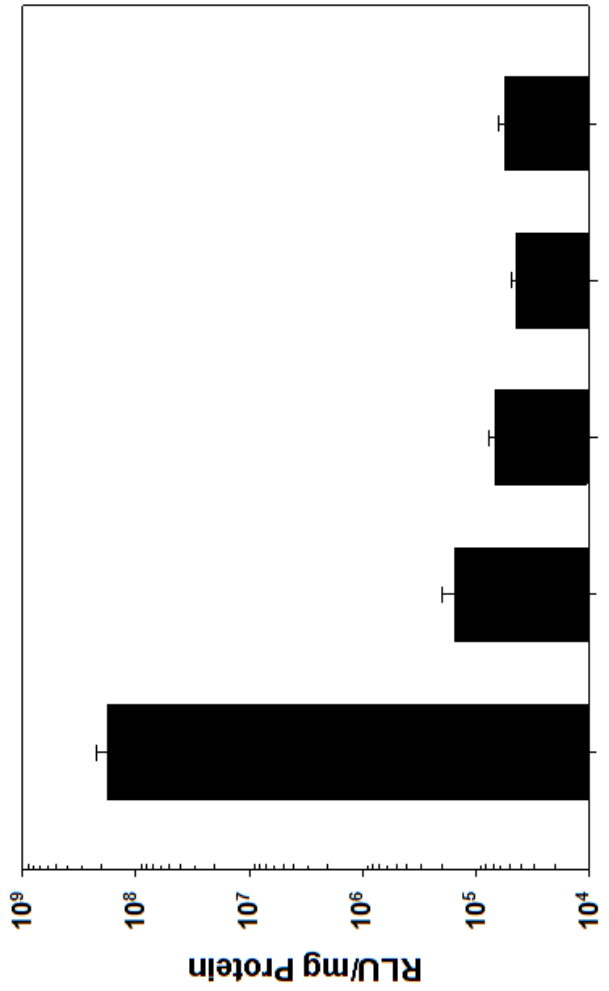

Figure 2.10: Transfection efficiency of micelle/pDNA complexes using MCF7 cells (n=3; mean ± SD)

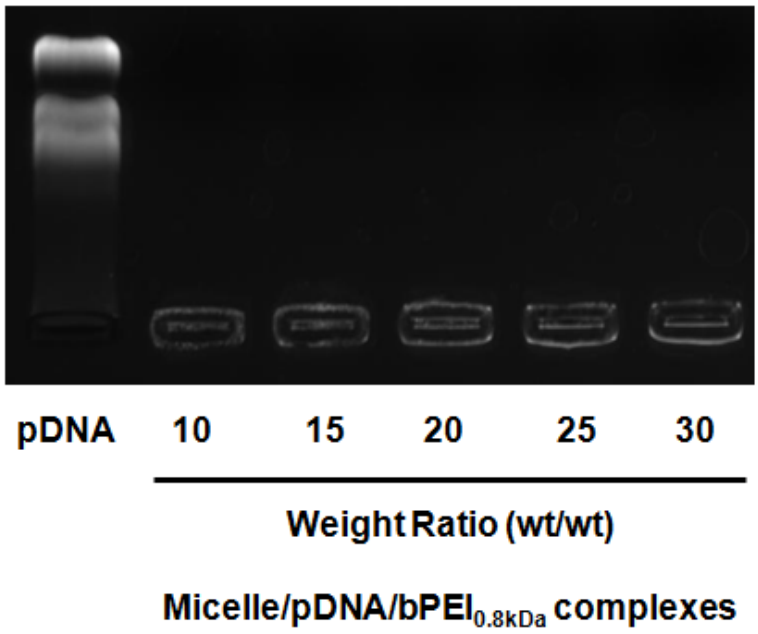

Figure 2.11: Surface shielding of micelle/pDNA complexes with 5 µg of $bPEI_{0.8kDa}$. Micelle/pDNA complexes were prepared using 1 µg of pDNA. (n = 3; mean ± SD)

Particle size and zeta potential of "fresh" micelle/pDNA complexes and "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complexes were further investigated to address whether $bPEI_{1.8kDa}$ mainly affected the surface shielding on the micelle/pDNA complexes or induced tighter complexation in the micelle/pDNA complexes. As shown in Fig. 2.12, the particle sizes of "fresh" micelle/pDNA complexes and "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complexes were not significantly different. This finding indicates that the major effect of $bPEI_{1.8kDa}$ treatment is probably not the induction of tighter complexation. However, the introduction of $bPEI_{1.8kDa}$ strongly increased the zeta potential to more positive values (30-40 mV) compared to micelle/pDNA complexes where the pDNA was fully exposed (approximately -20 mV at WR 1 to 2) or partially pDNA exposed (approximately 0-20 mV

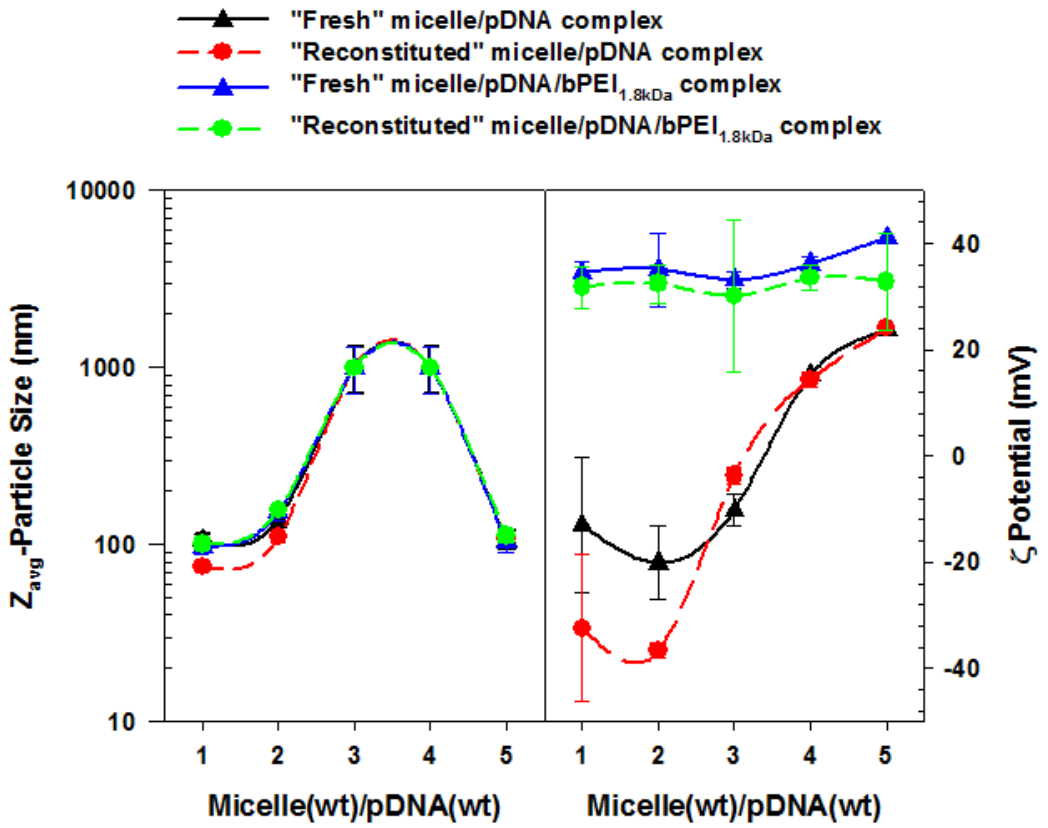

Figure 2.12: Particle size and zeta potential of micelle/pDNA complexes and micelle/pDNA/bPEI$_{1.8kDa}$ complexes before and after reconstitution. (n=5; mean ± SD)

at WR 3 to 5). These results may indicate that $bPEI_{1.8kDa}$ bound to and protected any pDNA exposed on the surface of micelle/pDNA complexes.

After examining the physicochemical similarities between "fresh" micelles and "reconstituted" micelles, the effects of reconstitution on polyplexes were characterized in terms of complexation, particle size, and zeta potential. Although EtBr-intercalated pDNA was detected for "fresh" micelle/pDNA complexes (WRs 4-5), EtBr-intercalated pDNA was not detectable for "reconstituted" micelle/pDNA complexes (WRs 4-5) (Fig. 2.7). It is possible that the lyophilization process may decrease the distance between phosphate groups in the pDNA and the primary amines of $bPEI_{25kDa}$ corona and thus further increase their electrostatic attraction. These tighter interactions could then prevent EtBr from intercalating with pDNA. However, in the case of $bPEI_{1.8kDa}$-shielded micelle/pDNA complexes, there was no apparent lyophilization effect because the polyplexes (WRs 1-5) had completely shielded pDNA regardless of whether the polyplexes were "fresh" or "reconstituted" (Fig. 2.7). Monitoring particle size of the polyplexes confirmed that the lyophilization and reconstitution process did not significantly affect particle size of "reconstituted" polyplexes compared to "fresh" polyplexes regardless of whether they were $bPEI_{1.8kDa}$-shielded or unshielded micelle/pDNA complexes (Fig. 2.12). In addition, the zeta potential of "reconstituted" polyplexes was not significantly different from "fresh" polyplexes (Fig. 2.12). These facts support the theory that the lyophilization and reconstitution of polyplexes may not negatively impact the physicochemical characteristics of "fresh" polyplexes.

2.4.4. Biological Characteristics of $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ Micelle/pDNA/$bPEI_{1.8kDa}$ Complexes

Transfection experiments using micelle/pDNA/$bPEI_{1.8kDa}$ complexes were performed in MCF7 breast cancer cells, using $bPEI_{25kDa}$/pDNA (N/P 5) complexes as a control. As shown in Fig. 2.13, transfection efficiencies for "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complexes (WRs 1-5) were much higher (50-100 fold) than for micelle/pDNA complexes (WRs 15-30) (Fig. 2.10. This indicates that the surface shielding conferred by the addition of $bPEI_{1.8kDa}$ may prevent bioactivity loss of pDNA via protection from nuclease-mediated degradation. Nevertheless, "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complexes (WRs 1-5) still had 20-40-fold lower transgene expression levels than "fresh" $bPEI_{25kDa}$/pDNA (N/P 5) complexes (Fig. 2.13).

Interestingly however, "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ complexes (WRs 1-5) had enhanced transfection efficiencies up to 16 times higher than the transfection values of their "fresh" micelle/pDNA/$bPEI_{1.8kDa}$ complex counterparts (WRs 1-5) (Fig. 2.13). This was especially true for micelle/pDNA/$bPEI_{1.8kDa}$ complexes that were less than 200 nm in diameter (*i.e.*, WRs 1, 2, and 5), where the process of lyophilization and reconstitution significantly enhanced their transfection efficiencies compared to their "fresh" polyplex counterparts ($p<0.001$). The transfection efficiency of "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ complexes (WR 1) was much closer (3.6 times lower) to transfection levels using "fresh" $bPEI_{25kDa}$/pDNA (N/P 5) complexes compared to "fresh"

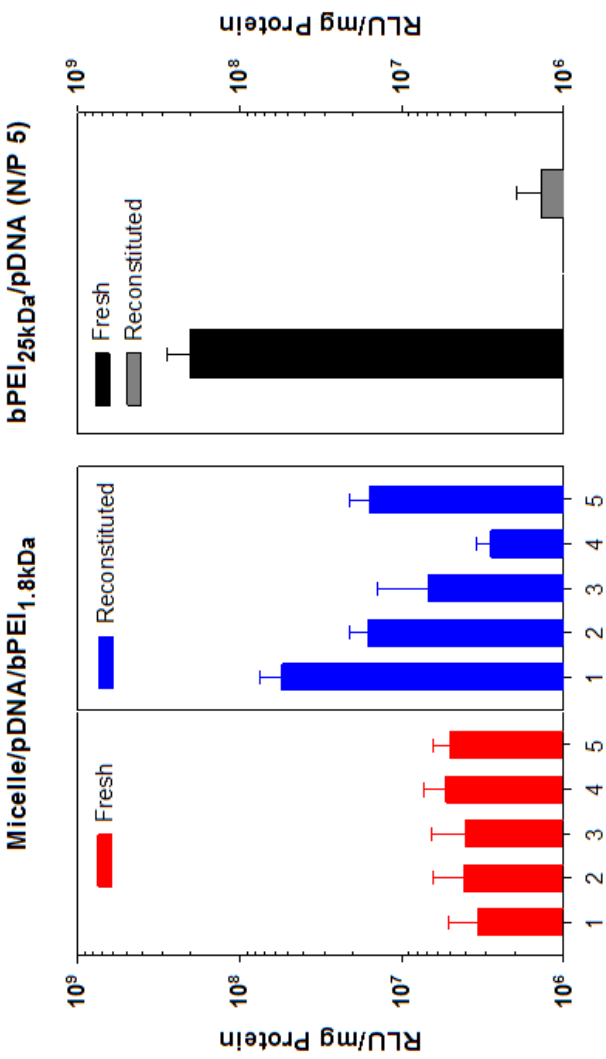

Figure 2.13: Transfection efficiency of fresh and reconstituted micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding). (n=8; mean ± SD)

micelle/pDNA/bPEI$_{1.8kDa}$ complexes (WR 1; 59-fold lower) (Fig. 2.13). Rather interestingly "reconstituted" micelle/pDNA/bPEI$_{1.8kDa}$ complexes (WR 1) had approximately 39-fold higher transfection efficiency than "reconstituted" bPEI$_{25kDa}$/pDNA (N/P 5) complexes prepared from sticky and lyophilized bPEI$_{25kDa}$/pDNA (N/P 5). Given the effect of reconstitution on bPEI$_{25kDa}$/pDNA complexes, the benefit of transfection enhancement for "reconstituted" micelle/pDNA/bPEI$_{1.8kDa}$ complexes (WR 1) may increase its appeal as a potential alternative to bPEI$_{25kDa}$ as a transfection reagent because the micelle system was also considerably less cytotoxic than bPEI$_{25kDa}$.

Although the micelle possesses more biocompatibility than bPEI$_{25kDa}$, polyplex cytotoxicity can also significantly affect transfection results. Thus, the cytotoxicity of "fresh" and "reconstituted" polyplexes (WRs 1-5) was evaluated in MCF7 cells and compared to the cytotoxicity of "fresh" bPEI$_{25kDa}$/pDNA complexes (N/P 5). As shown in Fig. 2.14, the cell viability of "fresh" micelle/pDNA complexes was over 90% compared to the untransfected control cells and was not statistically different from "reconstituted" micelle/pDNA complexes. Although bPEI$_{1.8kDa}$ was introduced into the micelle/pDNA complexes, the polyplexes still showed cell viability of approximately 90% regardless of whether they were subjected to lyophilization and reconstitution. Overall, the cytotoxicity of micelle-based polyplexes was significantly lower than "fresh" bPEI$_{25kDa}$/pDNA complexes (>90% versus <75%, $p<0.01$).

To investigate why "reconstituted" micelle/pDNA/bPEI$_{1.8kDa}$ (WR 1) complexes showed higher transfection efficiency than "fresh" micelle/pDNA/bPEI$_{1.8kDa}$ (WR 1) complexes but lower transfection efficiency than "fresh" bPEI$_{25kDa}$/pDNA (N/P 5)

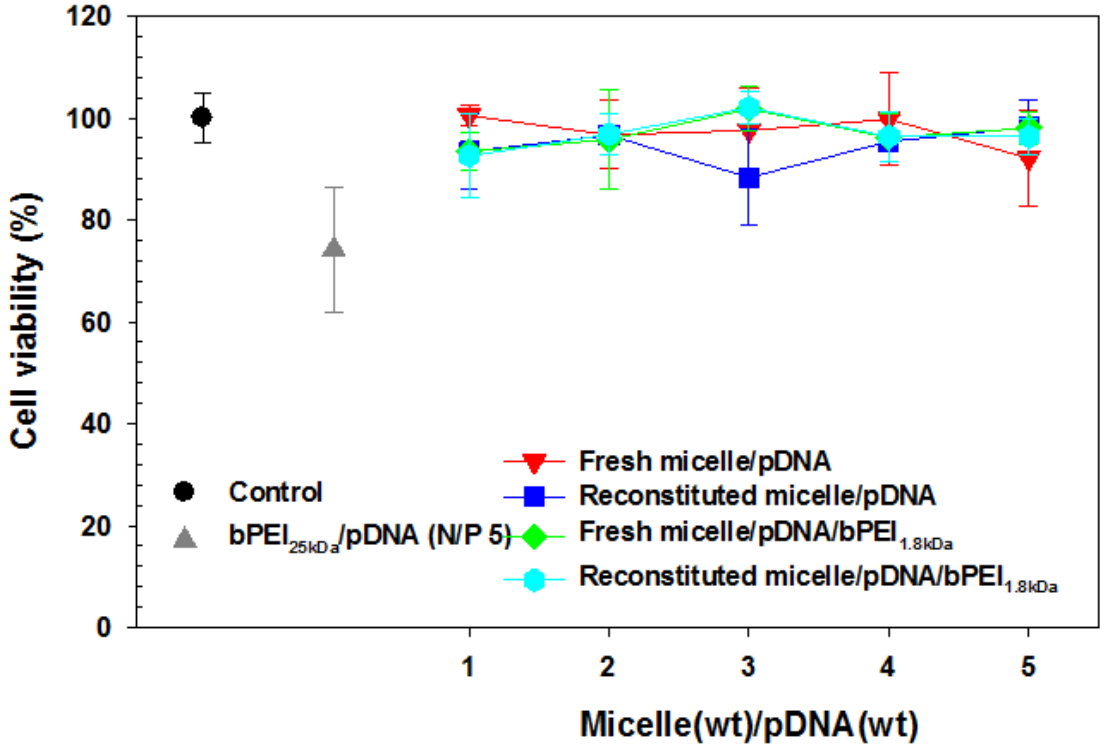

Figure 2.14: Cytotoxicity of fresh and reconstituted micelle/pDNA/$bPEI_{1.8kDa}$ complexes (0.5 µg of pDNA) in MCF7 cells (2.5×10^5 cells at seeding). (n=4; mean ± SD)

complexes, cellular uptake of complexed pDNA was analyzed in MCF7 cells. The number of DNA molecules per cell was quantified using flow cytometry. As shown in Fig. 2.15, from among the different polyplexes tested, "fresh" $bPEI_{25kDa}$/pDNA (N/P 5) showed the highest cellular uptake. "Reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ complexes had higher up-take compared to their "fresh" counterparts. These differences in the amount of intracellular pDNA support the benefit of reconstitution on increasing the transfection efficiency of micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1).

2.4.5. Scalability of $(PLGA_{36kDa})_2$-b-$bPEI_{25kDa}$ Micelle/pDNA/$bPEI_{1.8kDa}$ Complexes

While the transfection results using "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes appear very promising *in vitro*, *in vivo* and clinical applications of polymeric gene vectors would require large scale polyplex preparation with limited polyplex volume. In addition, one concern in gene therapy is whether transfection and cytotoxicity results obtained using small scale polyplex doses can be scaled up proportionately and translated to reflect the transfection and cytotoxicity associated with large scale polyplex doses. Thus, pDNA dose-dependent transfection and cytotoxicity studies of "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes were conducted in MCF7 cells to evaluate the effects of increasing pDNA doses.

MCF7 cells were transfected with "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes at varying final concentrations of pDNA ranging from 0.5-10 μg/mL (1-20 μg pDNA delivered to 500,000 cells at initial seeding). As shown in Fig. 2.16(a), transfection

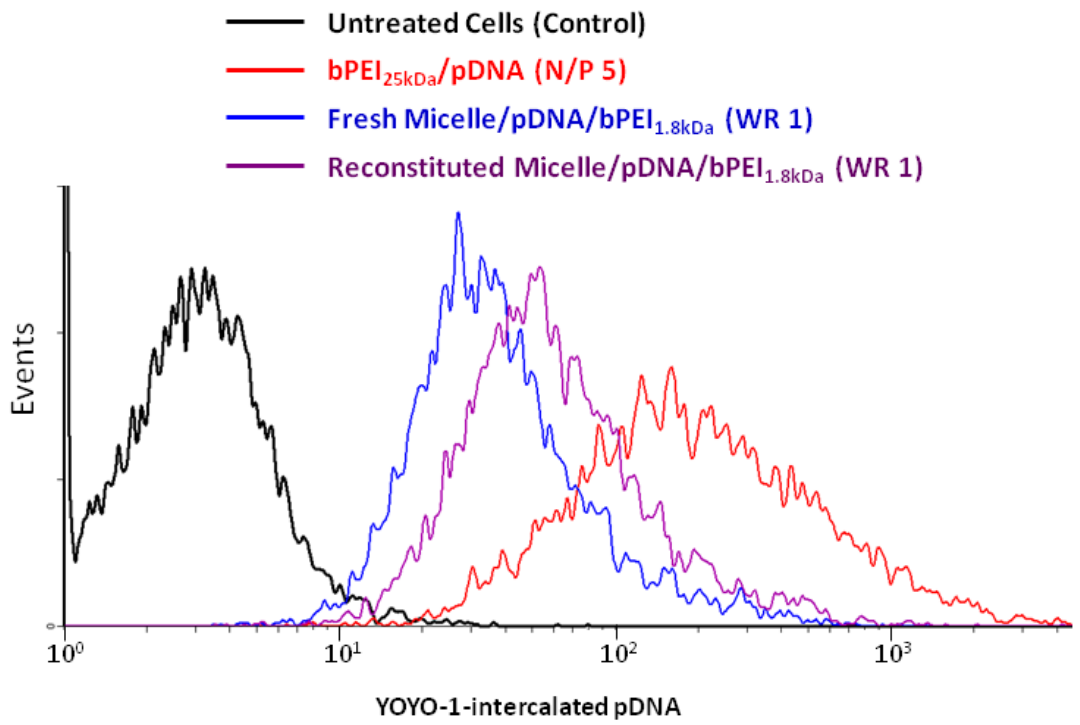

Figure 2.15: Cellular uptake of "fresh" and "reconstituted" micelle/pDNA/bPEI$_{1.8kDa}$ (WR 1) and "fresh" bPEI$_{25kDa}$/pDNA (N/P 5) complexes in MCF7 cells.

levels increased linearly with increasing pDNA dose; after accounting for cell viability, transfection increased nearly 50-fold with a pDNA dose of 20 μg (10 μg/mL) versus 1 μg (0.5 μg/mL). The cytotoxicity of micelle/pDNA/bPEI$_{1.8kDa}$ complexes was fairly low, with cell viabilities of 80% or higher at every pDNA concentration tested (Fig. 2.16(b)). Thus, this (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ polymeric micelle-based system could be used to deliver larger quantities of pDNA without causing undue toxicity.

Based on our initial findings, this micelle/pDNA/bPEI$_{1.8kDa}$ polyplex system has many favorable characteristics that make it an attractive gene delivery system. In addition, the structure of the particular copolymer used allows the possibility to bestow additional functionalities and further optimize the carrier. For instance, cationic polyplexes tend to have decreased colloidal stability due to nonspecific interactions with serum proteins and nontarget cells. This shortcoming could be overcome by conjugating poly(ethylene glycol) (PEG) which would reduce protein interactions and increase polyplex stability. Another option is to modify the carrier by conjugating targeting moieties to the polymeric micelle-surface which would enhance cellular internalization in the cell population of interest. Finally, because of the core-shell structure of our micelle system, there is the potential to utilize the core by loading it with hydrophobic therapeutic agents such as doxorubicin, dexamethasone or taxol among others, thus creating a dual-agent delivery system that can be lyophilized for storage and easily reconstituted when needed.

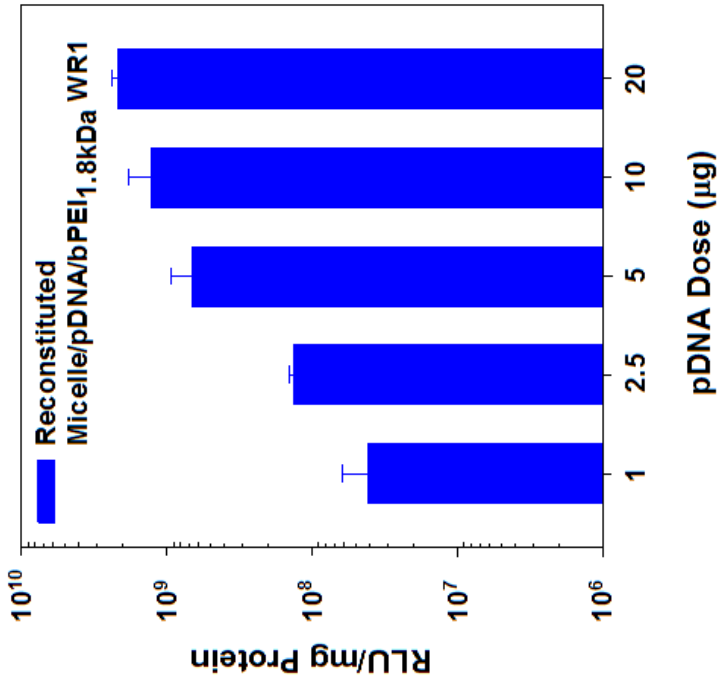

Figure 2.16: Effect of increasing pDNA dose on transfection efficiency and cytotoxicity in MCF7 cells (a) pDNA-dose dependent transfection efficiency of "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes in MCF7 cells. (5×10^5 cells at seeding) (n=4; mean ± SD)

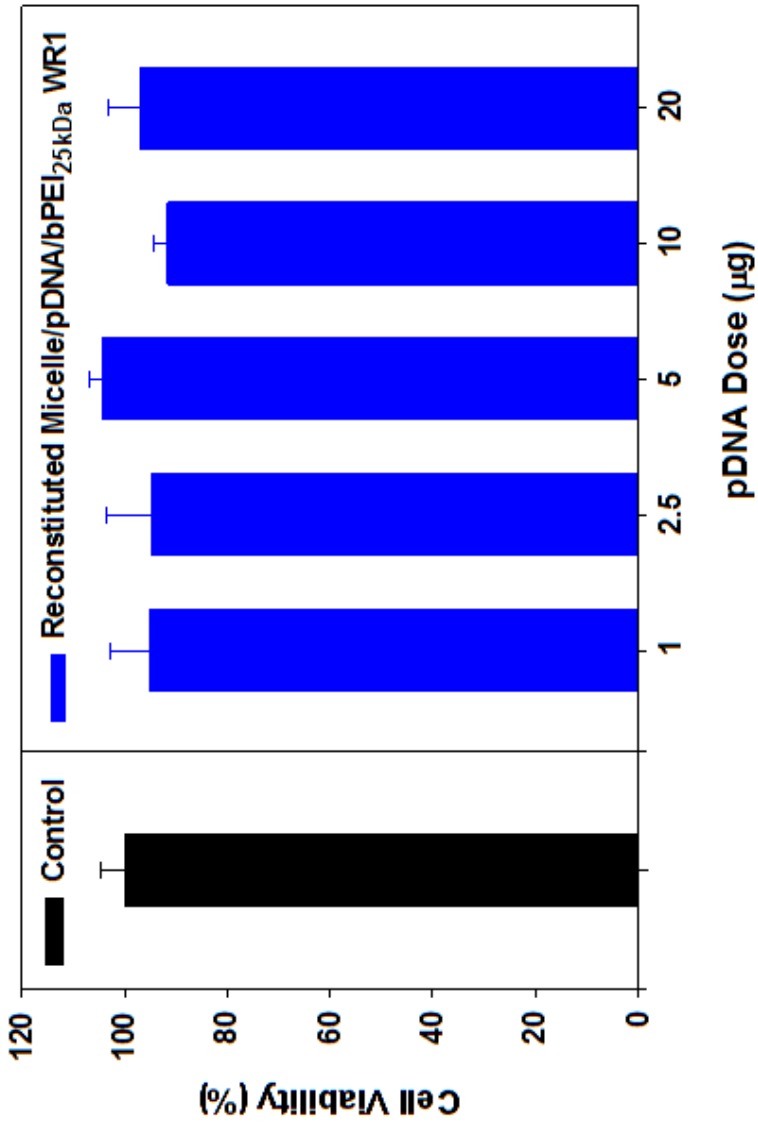

Figure 2.16 continued: (b) pDNA-dose dependent cytotoxicity of "reconstituted" micelle/pDNA/$bPEI_{1.8kDa}$ (WR 1) complexes in MCF7 cells (2.5×10^5 cells at seeding) (n=4; mean ± SD)

2.5. Conclusions

The goal of this study was to create a charged polymeric micelle-based system for gene therapeutics delivery that maintained efficacy following lyophilization and reconstitution. Using our $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ block copolymer, we were able to create a micelle system that remained stable following reconstitution. After complexation with pDNA, the micelle/pDNA system retained its particle size and zeta potential characteristics following lyophilization and reconstitution. Finally, the reconstituted system was able to successfully transfect breast cancer cells using a reporter gene with minimal toxicity to the cells. Although extrapolating the use of nonviral systems to treat human disease is still somewhat premature, we are encouraged by evidence from our lab showing the potential of this particular system as a reconstitutable charged polymeric micelle gene delivery system.

2.6. Acknowledgements

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2.7. References

[1] Louis A, Bartke A, Masternak MM. Effects of growth hormone and thyroxine replacement therapy on insulin signaling in Ames dwarf mice. J Gerontol A Biol Sci Med Sci 2010 April 2010;65(4):344-352.

[2] Pawlyk BS, Bulgakov OV, Liu X, Xu X, Adamian M, Sun X, et al. Replacement gene therapy with a human RPGRIP1 sequence slows photoreceptor degeneration in a murine model of Leber congenital amaurosis. Hum Gene Ther 2010;21(8):993-1004.

[3] Wang G, Qiu J, Wang R, Krause A, Boyer JL, Hackett NR, et al. Persistent expression of biologically active anti-HER2 antibody by AAVrh.10-mediated gene transfer. Cancer Gene Ther 2010;17(8):559-570.

[4] Zhou S, Mody D, DeRavin SS, Hauer J, Lu T, Ma Z, et al. A self-inactivating lentiviral vector for SCID-X1 gene therapy that does not activate LMO2 expression in human T cells. Blood 2010 May 10, 2010;116(6):900-908.

[5] López-Fraga M, Wright N, Jiménez A. RNA interference-based therapeutics: new strategies to fight infectious disease. Infect Disord Drug Targets 2008;8(4):262-273.

[6] Kang HC, Lee M, Bae YH. Polymeric gene carriers. Crit Rev Eukaryot Gene Expr 2005;15:317-342.

[7] Deyle D, Russell D. Adeno-associated virus vector integration. Curr Opin Mol Ther 2009;11(4):442-447.

[8] Bosticardo M, Ghosh A, Du Y, Jenkins NA, Copeland NG, Candotti F. Self-inactivating retroviral vector-mediated gene transfer induces oncogene activation and immortalization of primary murine bone marrow cells. Mol Ther 2009;17(11):1910-1918.

[9] Romano G, Marino I, Pentimalli F, Adamo V, Giordano A. Insertional mutagenesis and development of malignancies induced by integrating gene delivery systems: implications for the design of safer gene-based interventions in patients. Drug News Perspect 2009;22(4):185-196.

[10] Beer S, Bellovin DI, Lee JS, Komatsubara K, Wang LS, Koh H, et al. Low-level shRNA cytotoxicity can contribute to MYC-induced hepatocellular Carcinoma in Adult Mice. Mol Ther 2009;18(1):161-170.

[11] Kay MA. AAV vectors and tumorigenicity. Nat Biotechnol 2007 Oct;25(10):1111-1113.

[12] Wu TL, Ertl HC. Immune barriers to successful gene therapy. Trends Mol Med 2009 Dec 18;15:32-39.

[13] Li SD, Huang L. Non-viral is superior to viral gene delivery. J Control Release 2007 Nov 20;123(3):181-183.

[14] Ogris M, Wagner E. Tumor-targeted gene transfer with DNA polyplexes. Somat Cell Mol Genet 2002;27:85-95.

[15] Schatzlein AG. Targeting of synthetic gene delivery systems. J Biomed Biotechnol 2003;2003(2):149-158.

[16] Kang HC, Kim S, Lee M, Bae YH. Polymeric gene carrier for insulin secreting cells: Poly(l-lysine)-g-sulfonylurea for receptor mediated transfection. J Control Release 2005 May 7;105:164-176.

[17] Ferrer-Miralles N, Vazquez E, Villaverde A. Membrane-active peptides for non-viral gene therapy: making the safest easier. Trends Biotechnol 2008 May;26(5):267-275.

[18] Kang HC, Bae YH. pH-Tunable endosomolytic oligomers for enhanced nucleic acid delivery. Adv Funct Mater 2007;17(8):1263-1272.

[19] Chan CK, Jans DA. Using nuclear targeting signals to enhance non-viral gene transfer. Immunol Cell Biol 2002;80:119-130.

[20] Pouton CW, Wagstaff KM, Roth DM, Moseley GW, Jans DA. Targeted delivery to the nucleus. Adv Drug Deliv Rev 2007 Aug 10;59(8):698-717.

[21] Meng F, Hennink WE, Zhong Z. Reduction-sensitive polymers and bioconjugates for biomedical applications. Biomaterials 2009 Feb 4;30:2180-2198.

[22] Kim YH, Park JH, Lee M, Kim YH, Park TG, Kim SW. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J Control Release 2005 Mar 2;103(1):209-219.

[23] Kang HC, Kang H-J, Bae YH. A reducible polycationic gene vector derived from thiolated low molecular weight branched polyethyleneimine linked by 2-iminothiolane. Biomaterials 2011;32(4):1193-1203.

[24] Halama A, Kulinski M, Librowski T, Lochynski S. Polymer-based non-viral gene delivery as a concept for the treatment of cancer. Pharmacol Rep 2009;61(6):993-999.

[25] Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev 2001;47(1):113-131.

[26] Qiu L, Bae YH. Self-assembled polyethylenimine-graft-poly(e-caprolactone) micelles as potential dual carriers of genes and anticancer drugs. Biomaterials 2007;28(28):4132-4142.

[27] Wang Y, Ke C-Y, Weijie Beh C, Liu S-Q, Goh S-H, Yang Y-Y. The self-assembly of biodegradable cationic polymer micelles as vectors for gene transfection. Biomaterials 2007;28(35):5358-5368.

[28] Zhu C, Jung S, Luo S, Meng F, Zhu X, Park TG, et al. Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA-PCL-PDMAEMA triblock copolymers. Biomaterials 2010;31(8):2408-2416.

[29] Mishra V, Gupta U, Jain NK. Biowaiver: an alternative to in vivo pharmacokinetic bioequivalence studies. Pharmazie 2010;65(3):155-161.

[30] Fuchs GS, Mikkelsen S, Knudsen TK, Kappelgaard A-M. Ease of use and acceptability of a new pen device for the administration of growth hormone therapy in pediatric patients: An open-label, uncontrolled usability test. Clin Ther 2009;31(12):2906-2914.

[31] Collier J, Shah R, Gupta A, Sayeed V, Habib M, Khan M. Influence of formulation and processing factors on stability of levothyroxine sodium pentahydrate. AAPS PharmSciTech 2010;11(2):818-825.

[32] McCluskey SV, Graner KK, Kemp J, Aloumanis V, Ben M, Kupiec T, et al. Stability of fentanyl 5 {micro}g/mL diluted with 0.9% sodium chloride injection and stored in polypropylene syringes. Am J Health Syst Pharm 2009 May 1, 2009;66(9):860-863.

[33] Craig DQ. The mechanisms of drug release from solid dispersions in water-soluble polymers. Int J Pharm 2002;231(2):131-144.

[34] Bulmus V, Chan Y, Nguyen Q, Tran HL. Synthesis and characterization of degradable p(HEMA) microgels: use of acid-labile crosslinkers. Macromol Biosci 2007;7(4):446-455.

[35] Molina MdC, Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators. J Pharm Sci 2001;90(10):1445-1455.

[36] Flores-Fernández GM, Solá RJ, Griebenow K. The relation between moisture-induced aggregation and structural changes in lyophilized insulin. J Pharm Pharmacol 2009;61(11):1555-1561.

[37] Thompson C, Hansford D, Higgins S, Rostron C, Hutcheon G, Munday D. Preparation and evaluation of microspheres prepared from novel polyester-ibuprofen conjugates blended with non-conjugated ibuprofen. J Microencapsul 2009;26(8):676-683.

[38] Miene C, Klenow S, Veeriah S, Richling E, Glei M. Impact of apple polyphenols on GSTT2 gene expression, subsequent protection of DNA and modulation of proliferation using LT97 human colon adenoma cells. Mol Nutr Food Res 2009;53(10):1254-1262.

[39] Holzer M, Vogel V, Mäntele W, Schwartz D, Haase W, Langer K. Physico-chemical characterisation of PLGA nanoparticles after freeze-drying and storage. Eur J Pharm Biopharm 2009;72(2):428-437.

[40] Anhorn MG, Mahler H-C, Langer K. Freeze drying of human serum albumin (HSA) nanoparticles with different excipients. Int J Pharm 2008;363(1-2):162-169.

[41] Stark B, Pabst G, Prassl R. Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: Effects of cryoprotectants on structure. Eur J Pharm Sci 2010;41(3-4):546-555.

[42] Kuntsche J, Freisleben I, Steiniger F, Fahr A. Temoporfin-loaded liposomes: physicochemical characterization. Eur J Pharm Sci 2010;40(4):305-315.

[43] Miyata K, Kakizawa Y, Nishiyama N, Yamasaki Y, Watanabe T, Kohara M, et al. Freeze-dried formulations for in vivo gene delivery of PEGylated polyplex micelles with disulfide crosslinked cores to the liver. J Control Release 2005;109(1-3):15-23.

[44] Ou M, Xu R, Kim SH, Bull DA, Kim SW. A family of bioreducible poly(disulfide amine)s for gene delivery. Biomaterials 2009;30(29):5804-5814.

[45] Kang HC, Samsonova O, Bae YH. Trafficking microenvironmental pHs of polycationic gene vectors in drug-sensitive and multidrug-resistant MCF7 breast cancer cells. Biomaterials 2010;31(11):3071-3078.

[46] Silverstein RM, Bassler GC, Morrill TC. Spectrometric identification of organic compounds. 5th ed. Hoboken, New Jersey: John Wiley & Sons, 1991.

[47] Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV. Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. J Control Release 2007;119(1):77-85.

[48] Lee W-k, Park J-y, Yang EH, Suh H, Kim SH, Chung DS, et al. Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer. J Control Release 2002;84(3):115-123.

[49] Park TG. Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition. Biomaterials 1995;16(15):1123-1130.

[50] Braunecker J, Baba M, Milroy GE, Cameron RE. The effects of molecular weight and porosity on the degradation and drug release from polyglycolide. Int J Pharm 2004;282(1-2):19-34.

[51] Jalil R, Nixon JR. Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. J Microencapsul 1990;7(3):297-325.

CHAPTER 3

DEXAMETHASONE-LOADED CHARGED PLGA-*b*-PEI MICELLES FOR TARGETED NUCLEAR DELIVERY

3.1. Abstract

This study investigated the potential of dexamethasone to enhance the nuclear accumulation and subsequent gene expression of DNA delivered using a charged polymeric micelle-based gene delivery system. Three different copolymers having a PLGA-*b*-bPEI$_{25kDa}$ block co-polymer structure were synthesized and used to prepare cationic polymeric micelles loaded with dexamethasone; PLGA$_{36kDa}$-*b*-bPEI$_{25kDa}$ (PLGA:bPEI block ratio = 1), (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ (PLGA:bPEI block ratio = 2), and (PLGA$_{48kDa}$)$_2$-*b*-bPEI$_{25kDa}$ (PLGA:bPEI block ratio = 2). All three polymer systems retained proton-buffering ability of the bPEI$_{25kDa}$ block within the endosomal pH range. Particle size and zeta potential characteristics of dex-loaded micelles and dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ complexes were retained following reconstitution, so reconstituted complexes were prepared and used for all subsequent studies. Transfection levels using dex-micelle/pDNA/bPEI$_{1.8kDa}$ complexes were generally higher than their counterpart blank micelle/pDNA/ bPEI$_{1.8kDa}$ complexes, with the most dramatic increases in transfection levels using complexes with WR < 1. The dex-micelle/pDNA/bPEI$_{1.8kDa}$ systems showed very low cytotoxicity in MCF7 cells with cell

viabilities of 90% or higher for all conditions tested. These results suggest that this dexamethasone-loaded polymeric micelle-based system could be used to decrease the amount of polymer required for transfection, and as well as increase nuclear accumulation of the vehicle for gene and drug delivery applications.

3.2. Introduction

Gene therapy is a potential therapeutic strategy to treat diseases caused by missing or mutated genes. This can be accomplished by supplementing gene expression, replacing a mutated gene with a normal copy, or altering gene expression to correct phenotypes caused by defective genes [1-3]. The discovery that a genetic disease phenotype could be corrected by introducing exogenous DNA into a cell established the concept of human gene therapy [4-6] and since then numerous studies have been conducted in the quest to translate this knowledge into clinically relevant gene therapy applications. Some success has been achieved using viral-based delivery systems and lipid-based delivery systems and several clinical trials have been conducted using gene therapy to treat a variety of maladies [7-10]. Although viral-based systems have been able to attain high cell infection and gene expression rates, numerous concerns regarding their safety and immunogenicity have tainted their widespread development for clinical use and increased interest in developing nonviral delivery vectors [11-16].

Nonviral delivery systems are typically classified as lipid-based or polymer-based systems. Polymer-based systems in particular have many desirable features including excellent biocompatibility, no tumorigenicity and low immunogenicity. More importantly, polymer-based systems can easily be customized and adapted to suit a

particular system [12]. Polymers can be designed to include traits such as endosomal disruption [17, 18], decreased toxicity [19-21], and targeted-delivery to specific cell types [22, 23] or even the nucleus [24, 25]. Numerous polymer combinations have been designed and investigated as delivery systems. Block copolymer micelle systems specifically have been shown to modulate drug pharmacokinetics and prolong systemic circulation [26], making them a strong candidate for drug delivery applications. It has been shown that copolymers with a hydrophobic block-charged hydrophilic block composition such as poly(ε-caprolactone)-bPEI$_{1.8kDa}$ [27] or poly(lactide-*co*-glycolide)-*b*-bPEI$_{25kDa}$ [28] can naturally self-assemble into core-shell micelles, creating different compartments that can be loaded with hydrophobic drugs (core) and gene drugs (shell).

Despite the relative safety and immunogenicity of these carriers, polymer-based systems are plagued by low cell transfection and gene expression rates compared to levels attained using viral carriers. Numerous combinations of existing and novel polymer systems have been investigated to increase cell transfection using mechanisms such as engineering endosomal escape mechanisms, increasing cellular uptake through prolonged systemic circulation and targeting, and by enhancing nuclear penetration [29-32]. Increasing nuclear delivery is particularly important, as the gene has an increased chance of transfection and subsequent expression if it can enter the nucleus, which oftentimes is a limiting factor for successful transfection.

Transport into and out of the cell nucleus is highly regulated, and occurs through channels decorating the nuclear membrane called nuclear pore complexes [33]. Small molecules (diameter < 35nm, MW < 40kDa) such as water, glucose, and oxygen can

easily diffuse in and out of the channel, but larger molecules (diameter > 35nm, MW > 40kDa) require a nuclear localization signal and the aid of soluble nuclear transport factors in order to enter the nucleus [34]. Molecules that do not meet either of these two criteria are excluded from the nuclear compartment [35, 36]. While viruses have evolved specific mechanisms to overcome these natural cellular defenses and achieve nuclear delivery of their DNA with high efficiency, such abilities are sorely lacking in current polymeric-gene delivery candidates [37]. Methods to increase the nuclear delivery of therapeutic genes could augment gene transfection rates and enhance overall gene expression levels. This includes creating smaller polyplex particles that can easily diffuse through the nuclear pores, or conjugating nuclear localization signals or nuclear proteins to the carrier surface which would increase nuclear localization of the particles. Another option is to utilize receptor-mediated nuclear transport by identifying a receptor such as the glucocorticoid receptor and incorporating the corresponding ligands into the polyplex, thereby increasing nuclear accumulation and enhancing overall gene expression. One ligand of the glucocorticoid receptor in particular, dexamethasone, has been shown to have a dilatory effect on nuclear pores. Dexamethasone is a hydrophobic steroid hormone that upon binding to the glucocorticoid receptor causes translocation from the cytosol into the nucleus. It has been shown that cells treated with dexamethasone displayed transient dilation of the nuclear pore complexes which caused them to expand up to 135nm in diameter [34]. Others have demonstrated that the presence of dexamethasone facilitated the transport of polymer/DNA complexes into the nucleus, and conjugating dexamethasone

to the polymer increased transfection efficiency [38, 39]. Rebuffat *et al.* conjugated dexamethasone to DNA directly and evaluated gene transfer. They demonstrated that the dexamethasone-DNA conjugate clearly enhanced gene transfer, and this result was directly correlated to the presence of dexamethasone [40, 41]. As the nuclear entry of polymer/DNA complexes is currently hindered due to its larger diameter and lack of nuclear localization signals, dexamethasone could potentially be used to increase nuclear localization of polyplexes and subsequently enhance gene transfection efficiency and expression.

This study was designed to investigate the potential of using a charged polymeric micelle-based gene delivery system that showed enhanced nuclear delivery and increased gene expression. We had previously reported the creation of a micelle system based on a copolymer of branched polyethylenimine (bPEI) and poly(lactide-*co*-glycolide) (PLGA) ($(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$). The resulting copolymer self-assembled into a core-shell micelle structure and formed complexes with pDNA which could be lyophilized and reconstituted, and in fact achieved higher transfection efficiencies following reconstitution [28]. This copolymer along with two other bPEI-PLGA based polymers synthesized for this application ($PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ and $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$) were used to form micelles that were co-loaded with the hydrophobic drug dexamethasone within the core and a gene drug in the shell to form our delivery system. The resulting dexamethasone-loaded micelle/pDNA complexes were evaluated for particle size, zeta potential, dexamethasone-loaded content, pDNA condensation ability, nuclear localization, and cell transfection efficiency.

3.3. Materials and Methods

3.3.1. Materials

Poly(lactide-*co*-glycolide) (PLGA; Resomer®503H; lactide:glycolide=1:1 (mole/mole); approximate MW 36kDa) with a carboxylic group at one end and poly(lactide-*co*-glycolide) (PLGA; Resomer®504H; lactide:glycolide=1:1 (mole/mole); approximate MW 48kDa) with a carboxylic group at one end were purchased from Boehringer Ingelheim Pharm GmbH & Co. KG (Germany). Two branched polyethyleneimines having M_r 1.8kDa ($bPEI_{1.8kDa}$) and M_w 25kDa ($bPEI_{25kDa}$; M_n 10kDa) were purchased from Polysciences, Inc. (Warrington, PA) and Sigma-Aldrich Co. (St. Louis, MO) respectively. Fetal bovine serum (FBS), penicillin/streptomycin, and 0.25% trypsin/EDTA were purchased from Gibco BRL (Grand Island, NY). Triethylamine (TEA), *N*,*N*'-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), RPMI1640 cell culture medium powder, sodium bicarbonate, $_D$-glucose, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), human recombinant insulin, Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dexamethasone, and agarose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Luciferase assay kit was obtained from Promega Co. (Madison, WI). Spectrapor dialysis membrane MWCO 15kDa was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Ethidium bromide (EtBr) and the Biocinchoninic Acid Protein Assay Kit (BCA) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Plasmid DNA (pDNA)

encoded with the firefly luciferase reporter gene (gWiz-Luc) was purchased from Aldevron, Inc. (Fargo, ND).

3.3.2. Cell Culture

In this study, MCF7 cells (a human breast adenocarcinoma cell line) were used for determining the cytotoxicity of micelles and polyplexes, polyplex transfection and nuclear localization of polyplexes. All experiments used MCF7 cells cultured in RPMI1640 cell culture medium supplemented with insulin (4 mg/L), glucose (2 g/L), 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics at 37°C in humidified air containing 5% CO_2.

3.3.3. Synthesis and Characterization of Amphiphilic Copolymers

Several block copolymers composed of PLGA and $bPEI_{25kDa}$ ($(PLGA)_n$-*b*-$bPEI_{25kDa}$) were synthesized by conventional condensation between the carboxylic acid group from PLGA and the amine groups from $bPEI_{25kDa}$ following previously published methods [28]. In brief, PLGA (50µmol or 100µmol) and $bPEI_{25kDa}$ (50µmol) were dissolved separately in DMSO (100mL) and stirred independently for 4 hours. Then the two polymer solutions were mixed together along with DCC (1mmol), NHS (1mmol), and TEA (0.1mL) and polymerization was carried out at room temperature (RT) for 48 hours under constant stirring. About 100mL of deionized (DI) water was added to the reaction mixture and stirred for 2 hours; then the polymer solution was transferred to a dialysis tube (MWCO 15kDa) and dialyzed against DI water for 2 days to remove any unreacted $bPEI_{25kDa}$. Unreacted PLGA was removed by filtration at a later stage following micelle formation.

The dialysis solution was lyophilized to obtain the resulting block copolymers. The chemical structures were confirmed by ^{1}H-NMR spectroscopy.

3.3.4. Preparation and Characterization of Dexamethasone-Loaded Micelles

Micelles were prepared using standard dialysis techniques. For each $(PLGA)_n$-*b*-$bPEI_{25kDa}$ copolymer, the polymer was dissolved in DMSO at a concentration of 10mg/mL and stirred for 4 hours at RT. Dexamethasone was added to the polymer solution at a 1:1 weight ratio of polymer to drug, and the solution was stirred for an additional 2 hours. A volume of HEPES buffer (20mM, pH 7.4) equivalent to DMSO was added to the reaction mixture which was stirred for an additional 2 hours. The reaction mixture was transferred to a dialysis membrane (MWCO 15kDa) and dialyzed against HEPES 20mM pH 7.4 buffer at 4°C for 24 hours followed by dialysis against deionized water for an additional 6 hours to remove any residual salt. The resulting micelle solution was filtered using a 0.22µm filter to remove any unreacted PLGA remaining from the synthesis (which precipitates in water) and free dexamethasone (which also precipitates in water) and the remaining solution was lyophilized and stored protected from light at -20°C until needed.

Particle size and zeta potential of freshly-prepared and reconstituted dexamethasone-loaded micelles were measured in HEPES 20mM pH 7.4 buffer at room temperature using a Zetasizer $3000HS_A$ (Malvern Instruments Inc., Worcestershire, UK) with a fixed wavelength of 677nm and a constant angle of 90°.

Micelles were titrated using traditional acid-base titration methods to evaluate the amount of proton buffering capacity retained from the $bPEI_{25kDa}$ block in the various

$(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles [18, 21]. Each of the $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles were separately dispersed in 3mL of 150mM NaCl and titrated from pH 7.4 to pH 3 using 0.1N HCl. The proton buffering ability of the $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles was compared to $bPEI_{25kDa}$ alone within the pH range 7.4-5.1 because this pH range correlates with typical endolysosomal pHs. Buffering capacity (%) was calculated using the following equation [42]:

$$\text{Buffering capacity } (\%) = \frac{\Delta V_{HCl} \times C_{HCl}}{N} \times 100(\%)$$

where ΔV_{HCl} is the volume of the HCl solution (0.1N) that decreased the pH value of the polymer solution from pH 7.4 to pH 5.1, C_{HCl} is the concentration of the HCl solution (0.1N), and N is the total moles of protonable amine groups contained in the polymer.

The amount of dexamethasone loaded into the micelles was determined using UV spectroscopy. Prior to lyophilization, freshly prepared micelles were collected in pre-weighted falcon tubes. The volume of the bulk was measured, and a 200µL sample from each of the freshly prepared $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles was removed from the bulk and lyophilized separately. Following lyophilization, the final weight of the dried bulk micelles was measured and the mass of the dex-loaded micelles was determined. The separate 200µL sample was dissolved in 1mL of DMSO and stirred for 6 hours to ensure complete dissolution of the micelles. The absorbance of the micelle samples was measured using UV spectroscopy at 250nm wavelength. To create a standard curve, dexamethasone alone was dissolved in DMSO at several concentrations, stirred for 6

hours to ensure complete dissolution, and the absorbance of the samples was measured at 250nm. The amount of dexamethasone contained in the micelles was calculated based on the standard curve.

Cytotoxicity of dexamethasone-loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles was assessed using standard protocols for MTT-based cell viability assay [18, 21]. MCF7 breast cancer cells were seeded in 96-well plates at a density of 5000 cells/well in culture medium (0.1mL). Varying concentrations of sample solutions (10µL) were added to each well and incubated for 24 hours at 37°C in a cell culture incubator. Then MTT solution (10 µL, 5mg/mL) was added to each well and the plates were returned to the incubator for an additional 4 hours. Media was completely removed, 100µL of DMSO was added to each well, and the plates were incubated for 10 minutes at 37°C to allow the formazan metabolites to dissolve. Absorbance was measured at 570nm using a microplate reader (SpectraMax® M2, Molecular Devices, Sunnyvale, CA) and used to calculate cell viability.

3.3.5. Preparation and Characterization of Dexamethasone-Loaded Micelle/pDNA/$bPEI_{1.8kDa}$ Complexes

Micelle solutions were mixed with pDNA in HEPES buffer (20 mM, pH 7.4) containing 5% glucose (HBG) and incubated at RT for 30 minutes. Following complexation the polyplexes were mixed with a small amount of $bPEI_{1.8kDa}$ at a weight ratio of 2.5µg $bPEI_{1.8kDa}$ per 1µg pDNA. This method was used to create polyplexes for particle size and zeta potential measurements, gel retardation studies, gene transfection and cell viability tests.

Complexation between dexamethasone-loaded micelles and pDNA was evaluated by gel retardation and dye quenching methods [18, 23]. For the gel retardation studies, polyplex solution (0.5µg pDNA in 10µL) was loaded onto a 0.8% agarose gel containing EtBr (100ng/mL). Electrophoresis was run using 0.5X Tris-buffer containing boric acid and EDTA (TBE) at 100V for 60 minutes. The gel was imaged using an Alpha Innotech FluorChem FC2® instrument with Alpha View Software (Cell Biosciences, Santa Clara CA).

For the dye quenching assays, pDNA was mixed with ethidium bromide (EtBr) at a mole ratio of five nucleotides per one EtBr and pre-incubated in the dark for 30 minutes at RT. Using EtBr-intercalated pDNA, polyplexes were prepared following the same method described above. Polyplexes were excited at 515 nm and the emitted fluorescence was measured at 595 nm using a Spectramax® M2 spectraphotometer (Molecular Devices, Sunnyvale CA).

Particle size and zeta potential of polyplexes prepared using reconstituted dexamethasone-loaded micelles were measured at RT using a Zetasizer 3000HS_A (Malvern Instruments Inc., Worcestershire, UK) with a fixed wavelength of 677 nm and a constant angle of 90°.

In vitro transfection using reconstituted polyplexes was evaluated using MCF7 cells as previously reported [28, 43]. Transfection was evaluated using each of the three dex-loaded micelle systems separately to form complexes. A separate transfection experiment was also conducted using blank $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles and cells were co-treated simultaneously with the blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes and

free dexamethasone (0nM to 10μM) to confirm the effect of dexamethasone on gene transfection. Cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured for 24 hours in 2mL culture medium. One hour prior to transfection, the cell culture medium was removed and replaced with serum-free insulin-free culture medium (2 mL). Polyplexes were added to the wells (20 μL volume, pDNA content was fixed at 1 μg/well) and cells were incubated at 37°C for 4 hours. After the 4-hour transfection period, the serum-free medium was replaced with complete culture medium (supplemented with serum and insulin) and incubated for another 44 hours. Following incubation, culture medium was removed and cells were rinsed once with DPBS and lysed using a reporter lysis buffer. Luciferase gene expression (relative luminescence units (RLU)) was quantified by following the manufacturer's protocol for the luciferase assay. Protein content in the cells was evaluated by the BCA™ protein assay. Gene transfection efficiency is reported as RLU/mg protein.

In vitro cytotoxicity of reconstituted polyplexes was determined by MTT-based cell viability assay. The experimental procedure was the same as previously described for *in vitro* transfection except the cell number used (2.5×10^5 cells/well; 12-well plates) and the polyplex dose (10 μL; 0.5 μg pDNA/well). After completing the 48-hour transfection procedure, MTT solution (0.1 mL; 5 mg/mL) was added to the cells (in 1 mL of culture medium). After an additional 4-hour incubation, the MTT-containing medium was removed. The resulting formazan crystals produced by living cells were dissolved in 1mL of DMSO and absorbance was measured at 570 nm using a microplate reader [28].

The statistical significance of the data was evaluated by conducting unpaired Student's t-test with a confidence level of $p<0.05$, one-variable Analysis of Variance (ANOVA) and two-variable Analysis of Variance (ANOVA).

3.4. Results and Discussion

3.4.1. Synthesis and Characterization of $(PLGA)_n$-*b*-$bPEI_{25kDa}$ Amphiphilic Copolymers

Amphiphilic cationic block copolymers composed of $bPEI_{25kDa}$ and either $PLGA_{36kDa}$ or $PLGA_{48kDa}$ were synthesized through a conjugation reaction between the primary amines of $bPEI_{25kDa}$ and the monocarboxylated PLGAs (Fig. 3.1). Synthesis and block ratio of the copolymers (PLGA to $bPEI_{25kDa}$) were confirmed by ^{1}H-NMR. Following previously reported methods [28], the PLGA blocks were completely degraded into their lactic acid and glycolic acid subunits using 1M NaOH in D_2O in order to get an accurate reading of the block ratio between PLGA and $bPEI_{25kDa}$. Degradation products were completely soluble in the aqueous solution and based on the integration ratio of either lactic acid (or glycolic acid) and $bPEI_{25kDa}$, the block ratio of PLGA to $bPEI_{25kDa}$ was calculated to be 0.85 for $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ copolymer, 1.93 for $(PLGA_{36kDa})_2$-b-$bPEI_{25kDa}$ copolymer, and 2.38 for $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ copolymer (Table 3.1).

3.4.2. Preparation and Characteristics of Dexamethasone-Loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ Micelles

The amphiphilic nature of the block copolymers was used to form positively-charged dexamethasone-loaded micelles using conventional dialysis methods. The

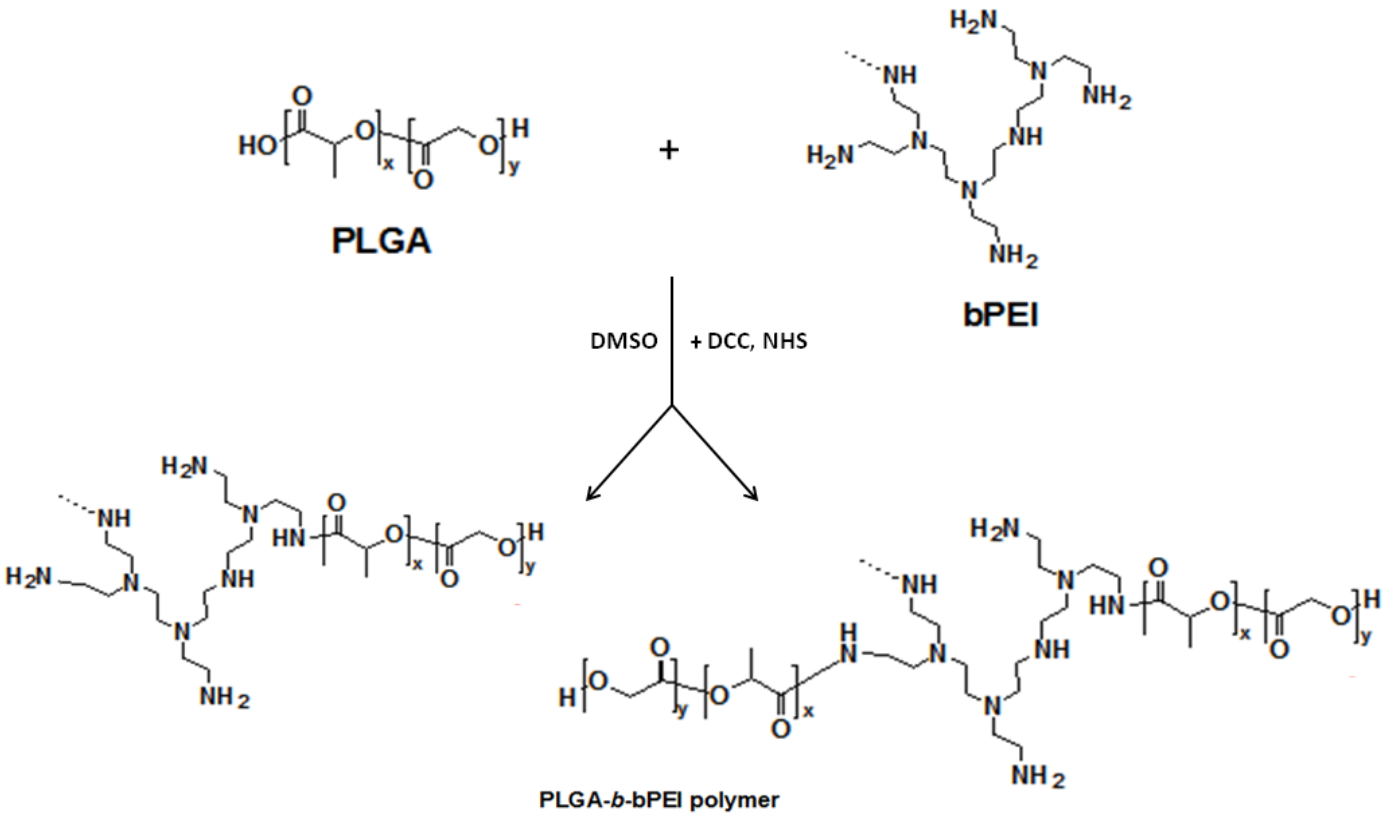

Figure 3.1: Reaction schematic for $(PLGA)_n$-*b*-$bPEI_{25kDa}$ copolymer

Table 3.1: Summary of $(PLGA)_n$-*b*-bPEI Block Copolymer Characteristics

	Feed Block Ratio (PLGA to $bPEI_{25kDa}$)	Actual Block Ratio (PLGA to $bPEI_{25kDa}$)	M_n of copolymer	Weight percent of $bPEI_{25kDa}$ in copolymer	Type of copolymer
$PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$	1	0.85	40.6 kDa	24.6%	Diblock
$(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$	2	1.93	79.5 kDa	12.6%	Triblock
$(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$	2	2.38	124.2 kDa	8.1%	Triblock

micelles assembled into a core-shell structure where dexamethasone was contained within the hydrophobic PLGA core and $bPEI_{25kDa}$ formed the hydrophilic shell (or corona). The average particle size diameter was 70-80 nm for cationic dexamethasone-loaded micelles made from $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$, 70 nm for cationic dexamethasone-loaded micelles made from $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$, and 80-100 nm for cationic dexamethasone-loaded micelles made from $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ (Z_{avg}-particle size relevant to hydrodynamic size) (Fig. 3.2). Correspondingly, the average zeta potential was 35-40 mV for dexamethasone-loaded micelles made from $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$, 35-45 mV for cationic dexamethasone-loaded micelles made from $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$, and 30-40 mV for cationic dexamethasone-loaded micelles made from $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ (Fig. 3.2). Particle size and zeta potential characteristics of micelles in aqueous solution were retained following reconstitution because the strong positively-charged corona prevented the formation of aggregated micelles due to charge repulsion. The relatively high molecular weight of PLGA used (MW 36kDa or 48kDa) endowed the micelle core with strong hydrophobicity [44-46] and slow degradation rate [44, 47, 48]. However, due to the presence of dexamethasone in the micelle core, storage of the dexamethasone-loaded micelles in aqueous solution would not be prudent because long-term exposure to changes in temperature, humidity, light, etc. could alter the physicochemical characteristics of the formulation and more importantly affect the biological function of the dexamethasone. We had previously found that $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles retained their physicochemical properties following lyophilization and reconstitution [28]. To determine whether this property extended to the

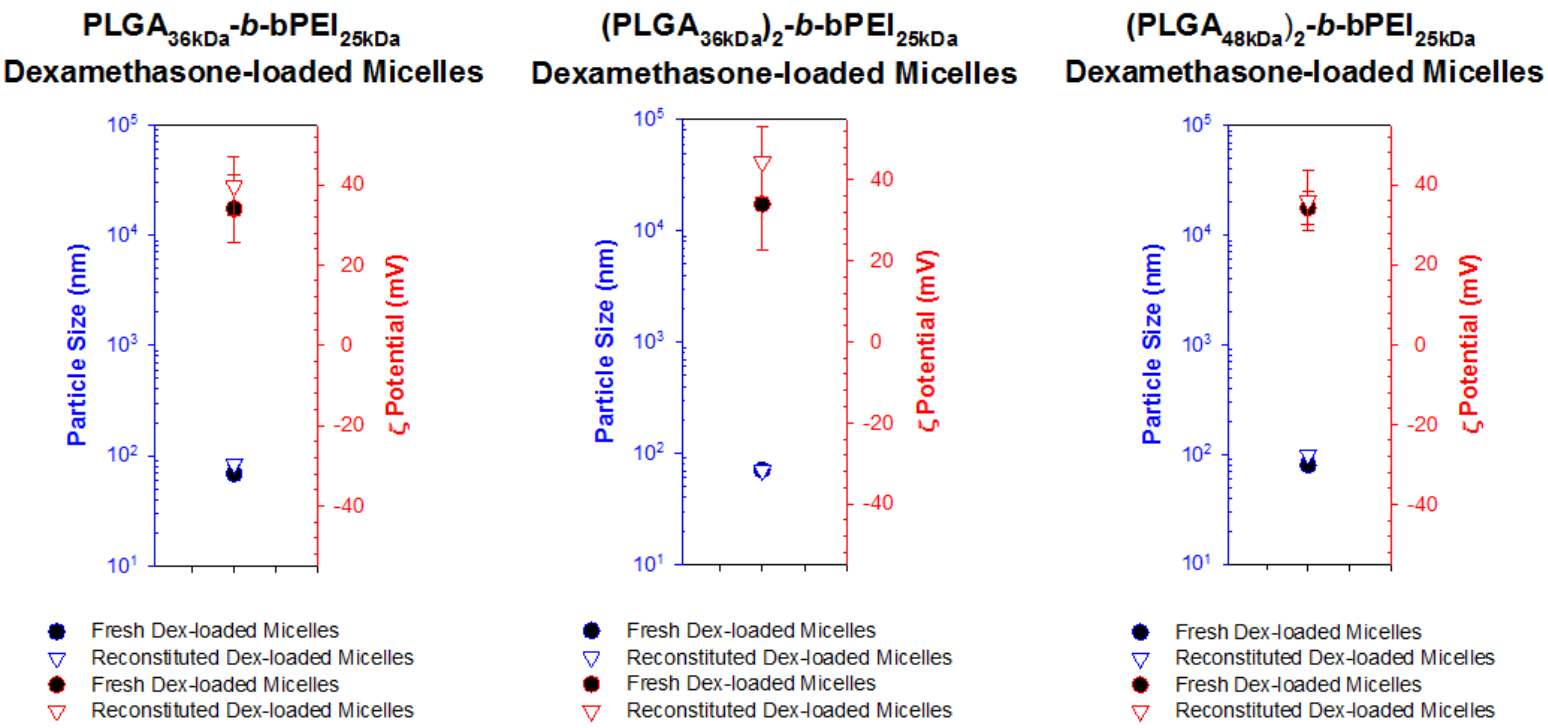

Figure 3.2: Particle size and zeta potential of dexamethasone-loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles before and after reconstitution. (n=4; mean ± SD)

dexamethasone-loaded micelle systems, particle size and zeta potential of all dexamethasone-loaded micelle formulations were monitored before and after lyophilization. As shown in Fig. 3.2, particle size and zeta potential for the reconstituted micelle systems were similar to those of the fresh micelle systems.

The proton-buffering capacity of $bPEI_{25kDa}$ has been well established. In order to determine how much proton-buffering ability was retained in each of the $(PLGA)_n$-*b*-$bPEI_{25kDa}$ dex-loaded micelles, micelles were dissolved individually in 3mL of 150mM NaCl at 0.5 mg/mL ($(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ = 3.28µmol in 3mL; $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ = 4.49µmol in 3mL; $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ = 7.57µmol in 3mL)and titrated from pH 7.4 to pH 3.0 by adding 0.1N HCl. Because the weight percentage of $bPEI_{25kDa}$ (based on M_n) in the copolymers varied ($(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$) = 9.7%, $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ = 12.6%, $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ = 22.3%), the buffering capacity was compared to $bPEI_{25kDa}$ at 0.5 mg/mL (3.49µmol in 3mL) and 0.75 mg/mL (5.23µmol amines in 3mL). As shown in Figure 3.3, all three dex-loaded micelle systems retained proton-buffering ability within the endosomal pH range (pH 5.1-7.4) although overall buffering capacity decreased following conjugation ($(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$) = 5.5%, $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ = 10.6%, $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ = 12.3%) compared to free $bPEI_{25kDa}$ alone (average 17.2% from the two different concentrations). The buffering capacity of the $bPEI_{25kDa}$ block was affected by both the block ratio of PLGA:bPEI (1:1 or 2:1) and the molecular weight of the PLGA block (MW 36kDa or 48kDa) due to the decreased ability of protons to access and protonate secondary or tertiary amines located near linkage between $bPEI_{25kDa}$ and the

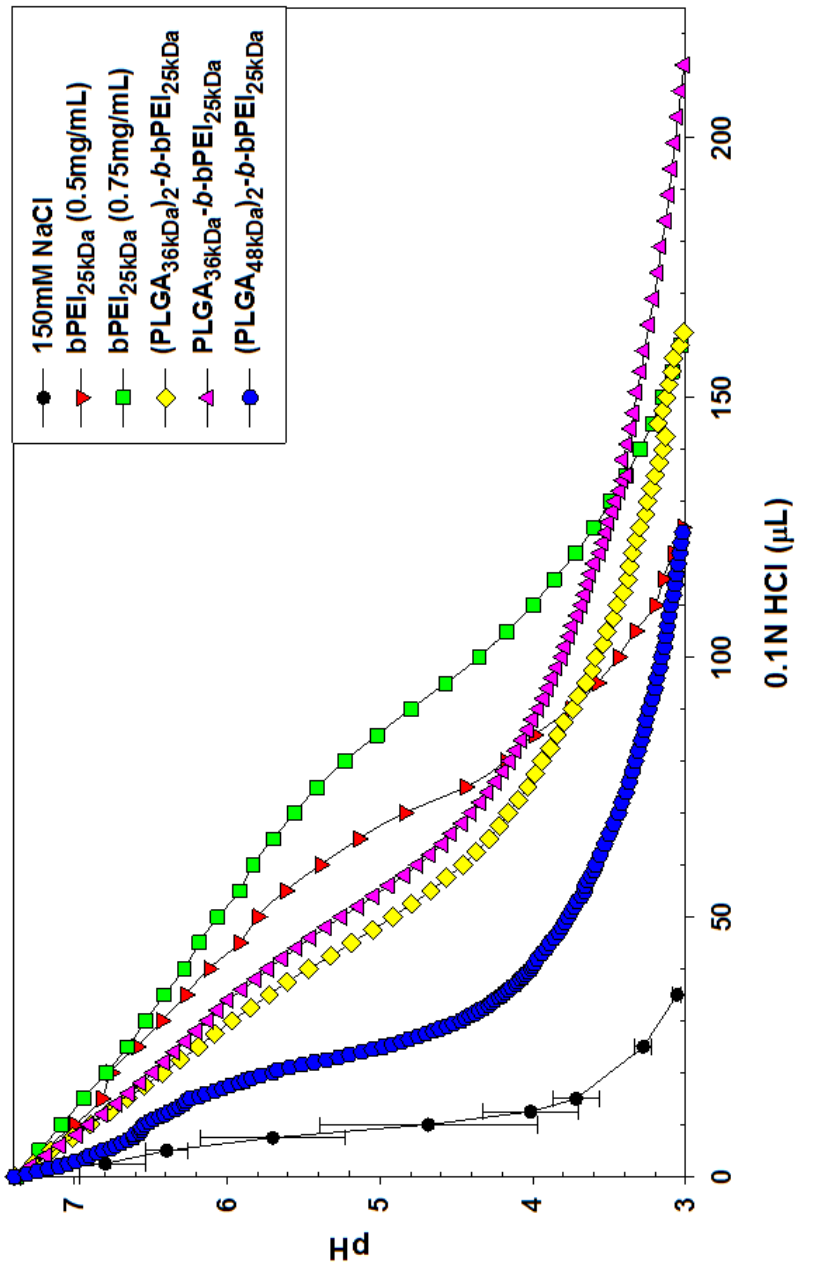

Figure 3.3: Proton buffering capacity of $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles and $bPEI_{25kDa}$. (n=3; mean ± SD)

strong hydrophobic PLGA block(s), which reduced the overall buffering ability of the copolymer [28].

The amount of dexamethasone incorporated into each of the micelle systems was determined by measuring the absorbance of dexamethasone at 250nm wavelength using standard UV spectroscopy. Dexamethasone content was determined to be 17.4wt% in dex-loaded $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ micelles, 7.6wt% in $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles, and 9.2wt% in $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$) micelles.

The toxicity of the dexamethasone-loaded polymeric micelles was compared to the toxicity of free dexamethasone and control polymers ($bPEI_{25kDa}$ and $bPEI_{1.8kDa}$). As expected, the dexamethasone-loaded polymeric micelles had quite low toxicity compared to $bPEI_{25kDa}$ alone (Fig. 3.4). The IC_{50} for all the polymers was greater than 200µg/mL, much higher than for $bPEI_{25kDa}$ alone (~12 µg/mL). The cytotoxicity contribution from the presence of dexamethasone in the core (100nM or less) and the concentration of the small amount of $bPEI_{1.8kDa}$ used in transfection studies (2.5 µg per 1 µg pDNA) was negligible; cell viabilities were unaffected by the presence of dexamethasone and higher than 90% for the concentration of $bPEI_{1.8kDa}$ used.

3.4.3. Preparation and Characteristics of Dexamethasone-loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ Micelle/pDNA/$bPEI_{1.8kDa}$ Complexes

Reconstituted dexamethasone-loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles were mixed with pDNA to form complexes and then coated with a small amount of $bPEI_{1.8kDa}$ to ensure that the pDNA was completely shielded (abbreviated as dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes). It had been previously determined that adding a

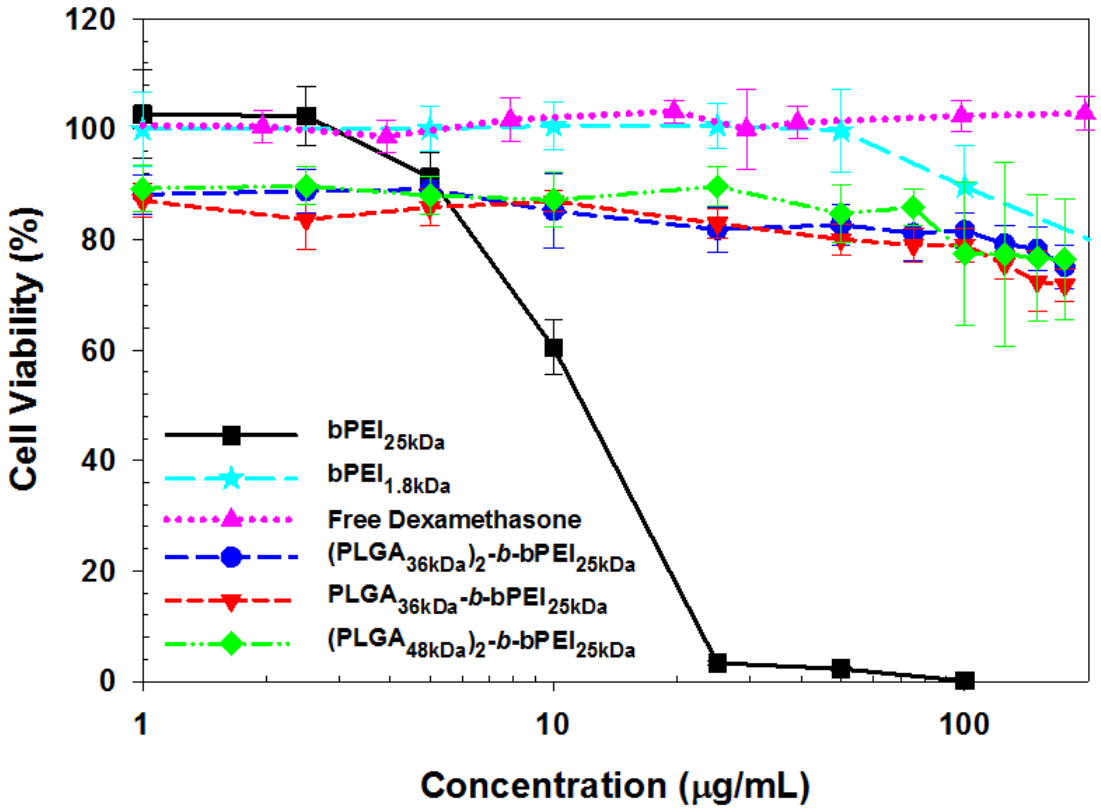

Figure 3.4: Cytotoxicity of $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelles, $bPEI_{25kDa}$, and $bPEI_{1.8kDa}$ in MCF7 cells. (n=6; mean ± SD)

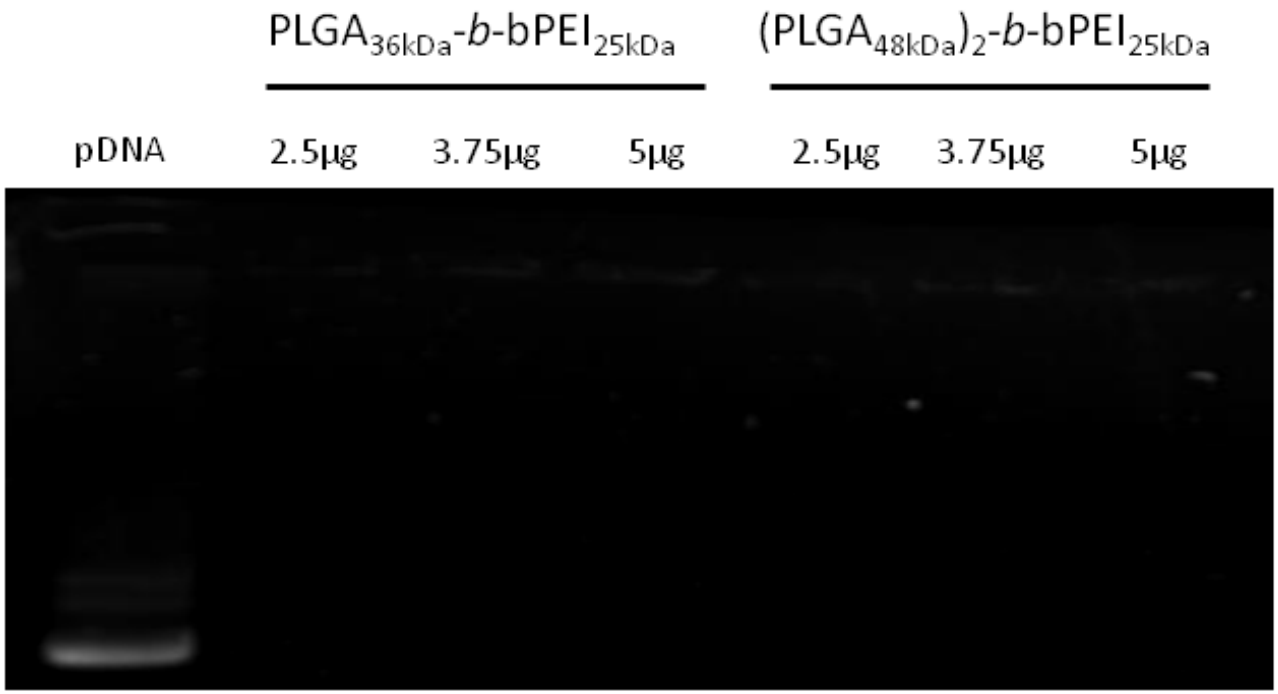

Figure 3.5: Surface shielding effect of $bPEI_{1.8kDa}$ on gene condensation of dex-micelle/pDNA complexes: agarose gel retardation. (n=3; mean ± SD)

small amount of $bPEI_{1.8kDa}$ (2.5µg per 1µg pDNA) completely shielded any exposed pDNA on the surface of micelle/pDNA complexes made using blank $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelles [28]. Since there were some differences in the composition of the three micelle systems it was confirmed that this amount of $bPEI_{1.8kDa}$ was sufficient to shield the pDNA in all dexamethasone-loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ micelle/pDNA complexes (Fig. 3.5). Having determined that the dexamethasone-loaded micelles retained their particle size and zeta potential characteristics following reconstitution, the next step was to confirm that dexamethasone-loaded micelle/pDNA/$bPEI_{1.8kDa}$ complexes remained complexed to the pDNA following reconstitution. Particle size, zeta potential and complexation of the polyplexes were monitored after reconstitution. As shown in Figure 3.6, pDNA remained shielded prior to (Figure 3.6a) and following lyophilization and reconstitution (Figure 3.6b), confirming that the reconstitution process did not negatively impact the physicochemical properties of polyplexes (Figure 3.6 a & b). In fact pDNA shielding increased following reconstitution as demonstrated by the decrease in fluorescence detected in the EtBr quenching assay (Fig. 3.7). Following this finding, only reconstituted complexes were used for all subsequent evaluation. Particle sizes of the reconstituted polyplexes were similar regardless of copolymer composition or weight ratio (100-180nm) and zeta potential remained strongly positive (30-40mV) (Figure 3.8). Previously we had shown that the particle size had increased considerably for blank $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ complexes at weight ratios 3 and 4 due to the formation of large aggregates [28], whereas in this study none of the dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes formed such large aggregates at the same weight

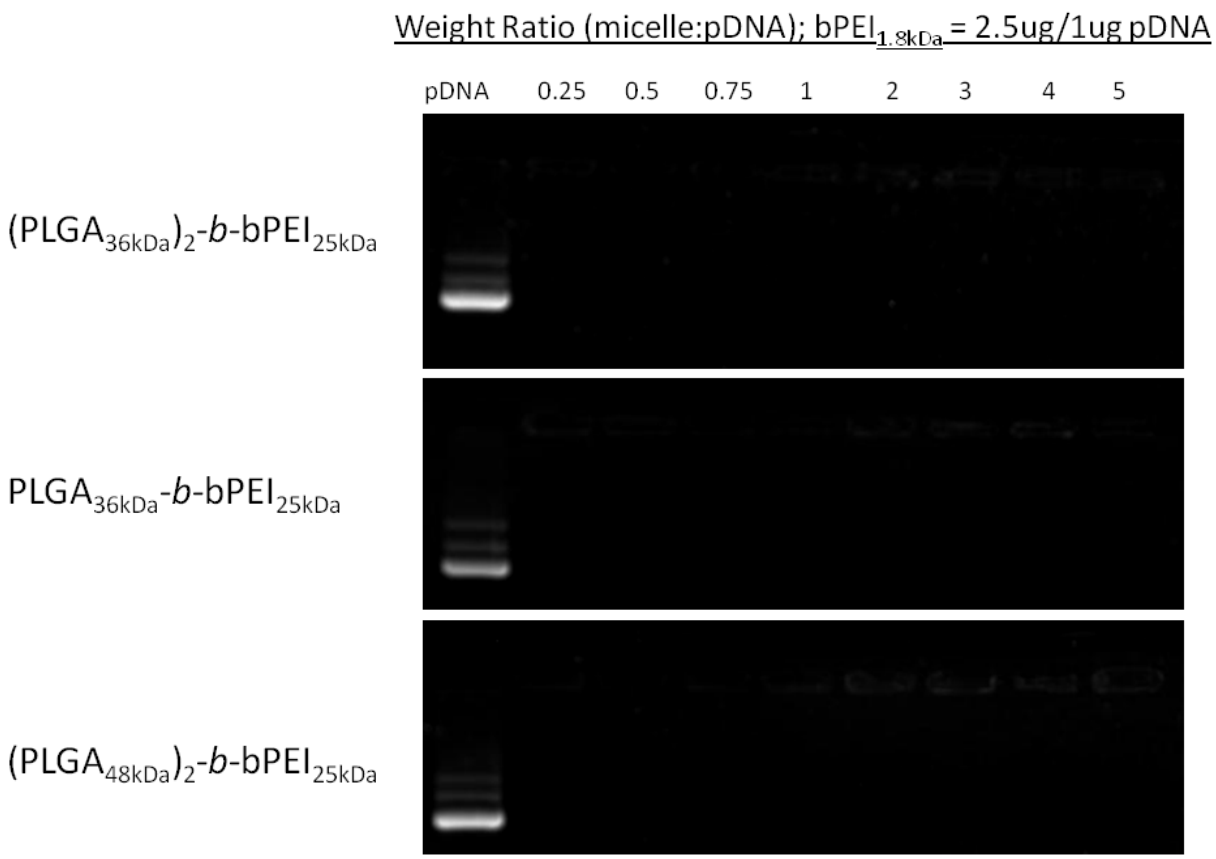

Figure 3.6: Gene condensation of dex-loaded micelle/pDNA complexes
(a) gene condensation of fresh dex-micelle/pDNA complexes: agarose gel retardation.

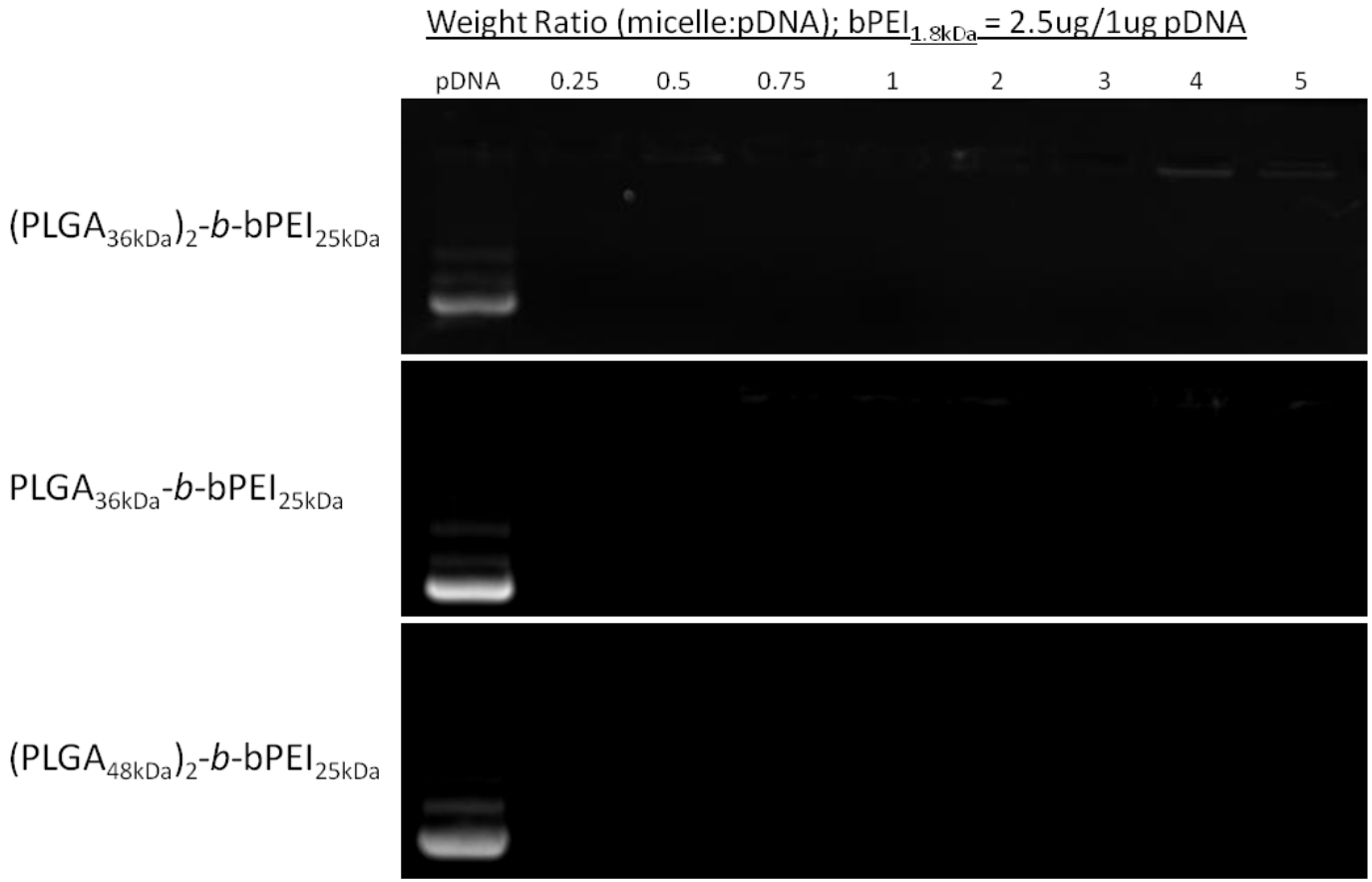

Figure 3.6 continued (b) gene condensation of reconstituted dex-micelle/pDNA complexes: agarose gel retardation.

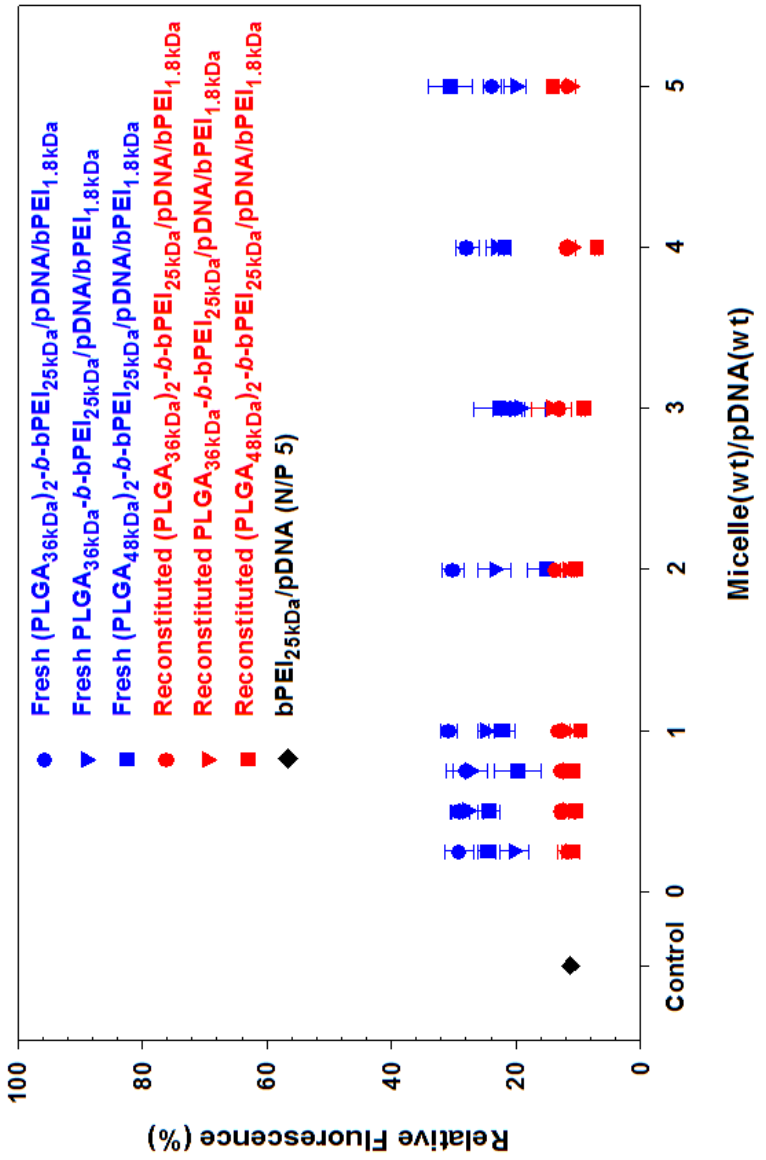

Figure 3.7: Gene condensation of fresh and reconstituted dex-micelle/pDNA complexes: dye quenching assay. (n=3; mean ± SD)

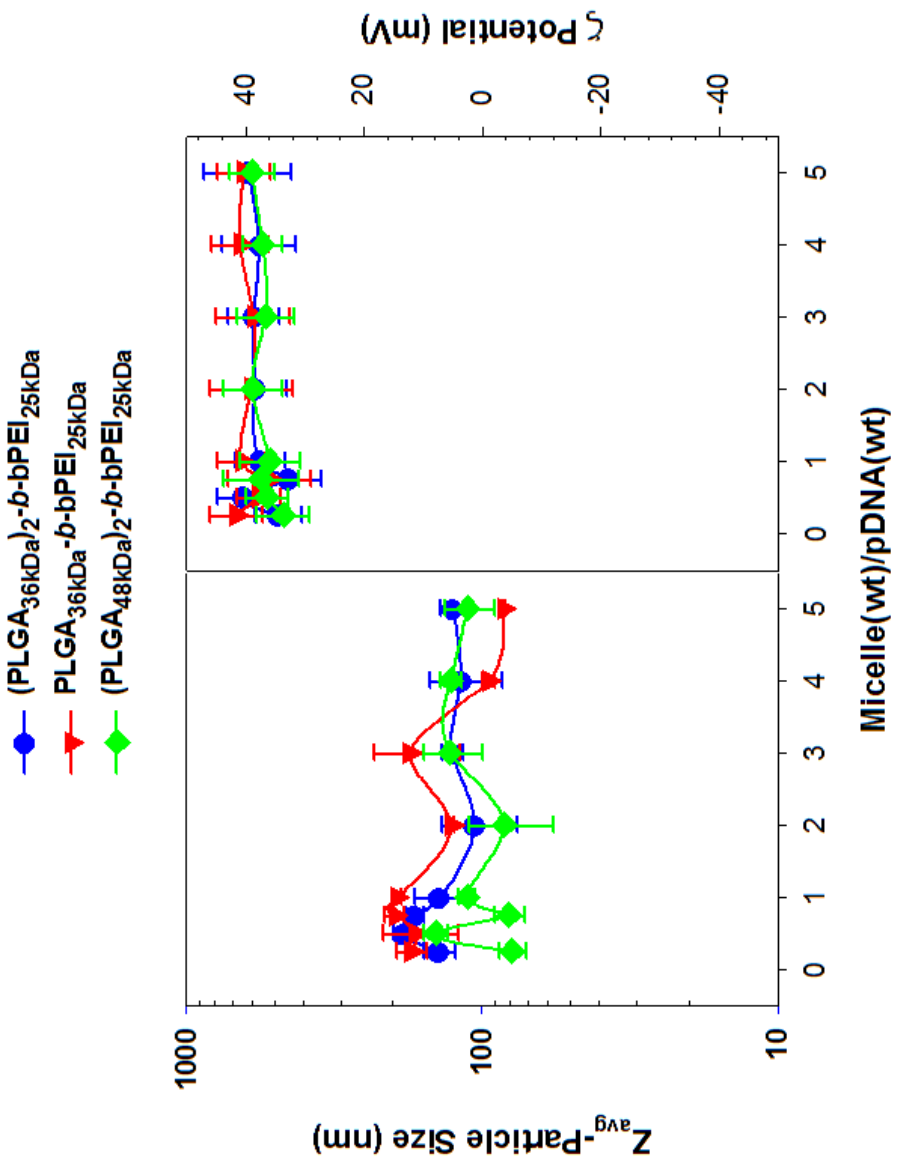

Figure 3.8: Particle size and zeta potential of reconstituted dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes. (n=3; mean ± SD)

ratios (3 and 4). This is probably due to the difference in zeta potential between the two micelle groups. The blank micelles had a zeta potential of 15-25mV, whereas the dex-loaded micelles had zeta potentials of 30-45mV. This stronger positive value most likely prevented the formation of large aggregates during complexation due to stronger charge repulsion between the micelles.

3.4.4. Biological Characteristics of Dexamethasone-Loaded $(PLGA)_n$-*b*-$bPEI_{25kDa}$ Micelle/pDNA/$bPEI_{1.8kDa}$ Complexes

Transfection experiments were performed in MCF7 breast cancer cells using reconstituted dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ complexes and compared to reconstituted blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes and fresh or reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes (Figure 3.9 (a-i)). Transfection efficiencies achieved using the dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ systems were slightly lower than fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes, although expression levels were fairly close for all weight ratios tested, ranging from only 1.4-fold to 4.8-fold lower expression, even for polyplexes with weight ratio (WR) < 1 (Figure 3.9(a), 3.9(d), 3.9(g)). Interestingly, one complex condition surpassed $bPEI_{25kDa}$/pDNA (N/P 5) transfection levels; $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ WR 3 complexes achieved 3.3-fold higher expression compared to $bPEI_{25kDa}$/pDNA (N/P 5) control complexes (Figure 3.9(a)). Since there was only one PLGA block in this particular co-polymer (diblock structure), the $bPEI_{25kDa}$ block was less affected by the conjugation and was more effective in aiding endosomal escape and gene transfection compared to the other two triblock copolymers which had two PLGA blocks each. This was more evident after comparing transfection levels using the

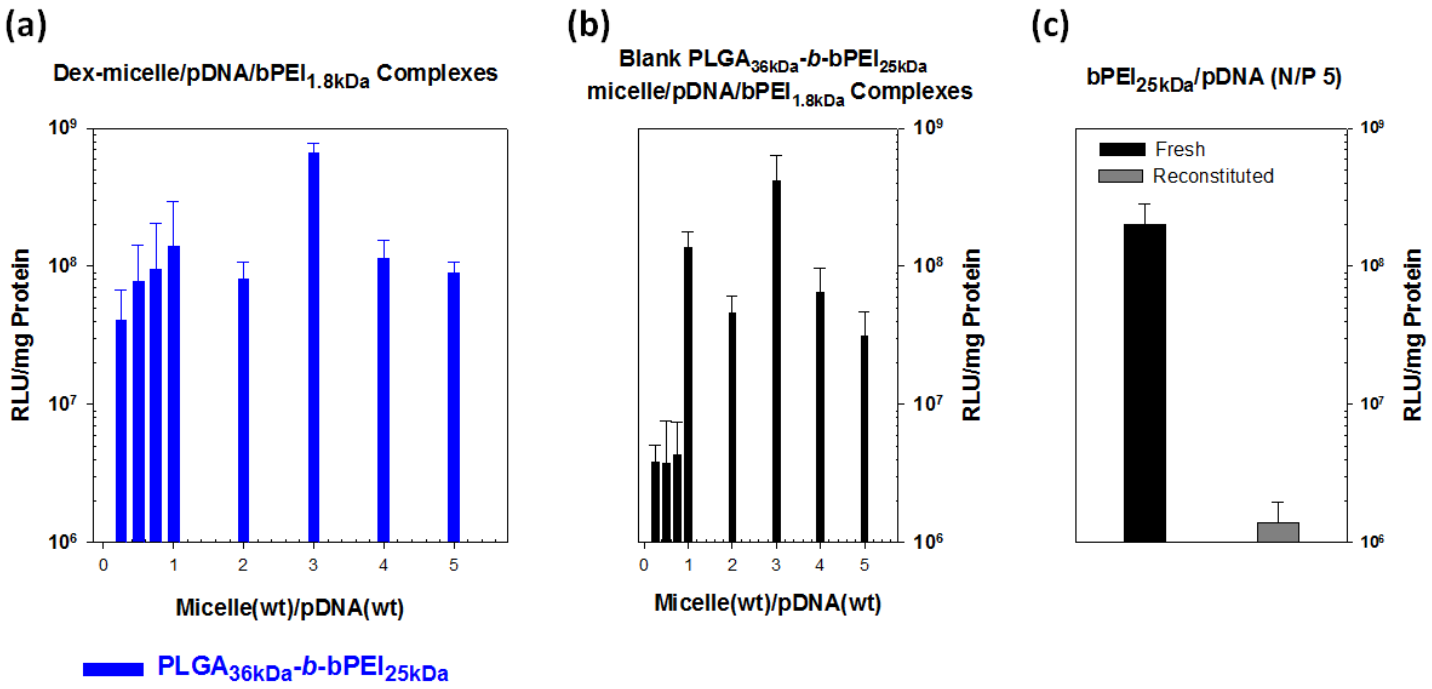

Figure 3.9: Transfection efficiencies of reconstituted dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes (a) Transfection efficiency of reconstituted $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (b) reconstituted $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (c) fresh and reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. (n=4; mean ± SD)

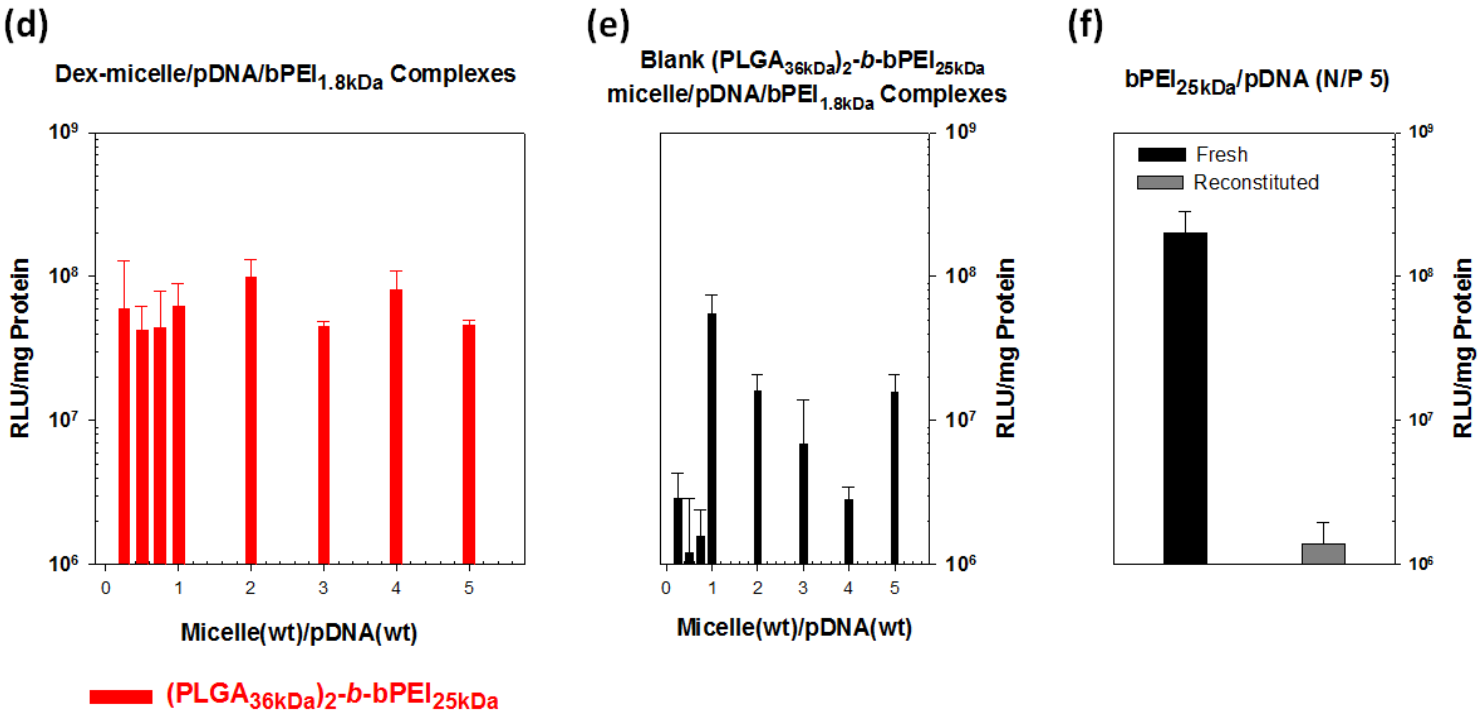

Figure 3.9 continued: (d) Transfection efficiency of reconstituted $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (e) reconstituted $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (f) fresh and reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. (n=4; mean ± SD)

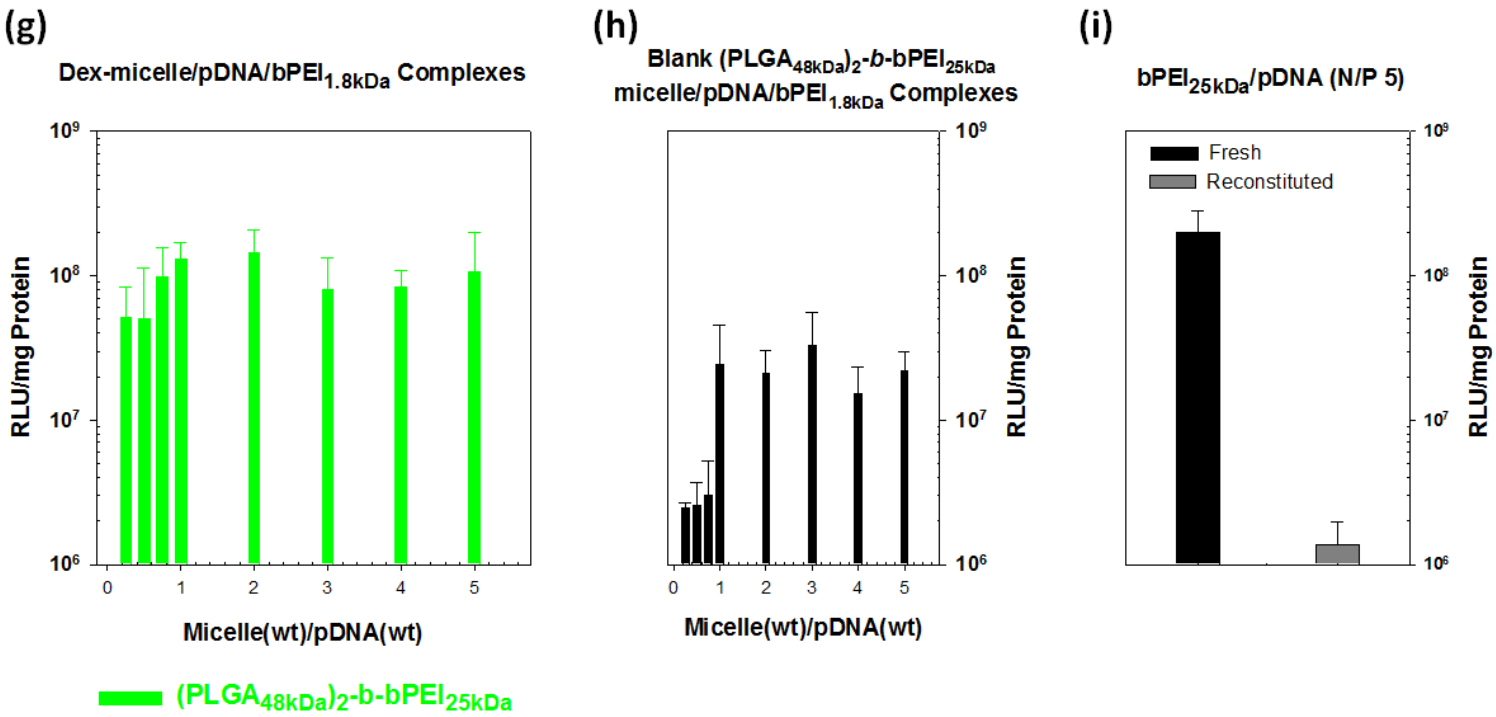

Figure 3.9 continued: (g) Transfection efficiency of reconstituted $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (h) reconstituted $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes (1 µg of pDNA) in MCF7 cells (5×10^5 cells at seeding), (i) fresh and reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. (n=4; mean ± SD)

blank micelle/pDNA/bPEI$_{1.8kDa}$ complexes, where the difference in transfection levels between blank diblock PLGA$_{36kDa}$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ complexes WR 1-5 was between 0.5-fold to 6.4-fold lower than fresh bPEI$_{25kDa}$/pDNA (N/P 5) complexes (Figures 3.9(a) and 3.9(c)), compared to the other two blank triblock micelle systems which contained two PLGA blocks each; transfection levels using blank micelle/pDNA/bPEI$_{1.8kDa}$ WR 1-5 complexes ranged from 3.7-fold to 71-fold lower than fresh bPEI$_{25kDa}$/pDNA (N/P 5) complexes (Figures 3.9(d), 3.9(f), 3.9(g), and 3.9(i), a much broader range than the diblock system.

The benefit of dexamethasone became obvious after comparing gene expression levels achieved using dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ complexes to control reconstituted blank micelle/pDNA/bPEI$_{1.8kDa}$ complexes and reconstituted bPEI$_{25kDa}$/pDNA (N/P 5) complexes. Transfection efficiencies using the diblock copolymer (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ complexes did not increase significantly using the dex-loaded micelles for weight ratios > 1 ($p > 0.29$), indicating that the amount of dexamethasone present was not enough to further enhance the transfection achieved from the bPEI block. However, the presence of dexamethasone significantly improved gene transfection efficiency for the triblock polymers, using (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ ($p < 0.00001$) or (PLGA$_{48kDa}$)$_2$-*b*-bPEI$_{25kDa}$/pDNA/bPEI$_{1.8kDa}$ complexes ($p < 0.0001$) from 1.1-fold up to 35-fold higher than their blank counterparts. Indeed the most dramatic improvement in gene transfection occurred for complexes with WR < 1. All three copolymer systems showed marked increase in gene transfection using dex-micelle/pDNA/bPEI$_{1.8kDa}$ WR < 1 complexes

compared to blank micelle/pDNA/bPEI$_{1.8kDa}$ WR < 1 complexes: 10.7-fold to 21.9-fold higher transfection levels for PLGA$_{36kDa}$-*b*-bPEI$_{25kDa}$ /pDNA/bPEI$_{1.8kDa}$ complexes ($p < 0.007$) (Figures 3.9(a) and 3.9(b)), 19.6-fold to 32.7-fold higher transfection levels for (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ complexes ($p < 0.0006$) (Figures 3.9 (d) and 3.9(e)), and 20.8-fold to 35-fold higher transfection levels for (PLGA$_{48kDa}$)$_2$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ complexes ($p < 0.008$) (Figures 3.9(g) and 3.9(h)), indicating that the presence of dexamethasone was beneficial in enhancing transfection levels. This could be very useful as a method to decrease the amount of polymer carrier necessary to achieve equivalent gene expression; decreasing the amount of polymer administered would also diminish unwanted toxicity resulting from the carrier, making these dex-loaded micelle carriers a favorable candidate for enhanced gene delivery to the nucleus.

The appeal of this system becomes even more apparent after comparing transfection levels using dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ complexes and reconstituted bPEI$_{25kDa}$/pDNA (N/P 5) complexes. Gene expression levels ranged from 30-fold to 44-fold higher using dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ WR 0.25 complexes compared to reconstituted bPEI$_{25kDa}$/pDNA (N/P 5) complexes, and increased up to 100-fold higher expression for dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ WR 1 complexes (Figure 3.9(a), 3.9(c), 3.9(d), 3.9(f), 3.9(g), and 3.9(i)). Previously we had shown that blank (PLGA$_{36kDa}$)$_2$-*b*-bPEI$_{25kDa}$ micelle/pDNA/bPEI$_{1.8kDa}$ WR 1 complexes were able to achieve transfection levels 39-fold higher than reconstituted bPEI$_{25kDa}$/pDNA (N/P 5) complexes. Thus the presence of dexamethasone increased expression levels an additional 60-fold

higher than the blank system, establishing that nuclear accumulation of polyplexes increased with the presence of dexamethasone in the micelle core. As it is known that $bPEI_{25kDa}$ is quite sticky and does not reconstitute well following lyophilization, the blank $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelle system had already proved to be a vast improvement, and the addition of dexamethasone improved the system even further. Curiously, transfection efficiency using $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ WR 3 complexes was an astounding 477-fold higher than reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes (Figure 3.9(a) and 3.9(c)). It is apparent that combining the transfection enhancement afforded by using reconstituted PLGA-*b*-bPEI micelle/pDNA/$bPEI_{1.8kDa}$ complexes coupled with the presence of dexamethasone in the core greatly increased gene transfection efficiency and subsequent gene expression in MCF7 cells.

It has been shown that the presence of dexamethasone (either as a pre-treatment or by conjugation) enhances the transfection of genes in cells. Dexamethasone binds to the glucocorticoid receptor in the cytosol of the cell, and then translocates into the nucleus. This means that if dexamethasone is conjugated onto the carrier surface, carrier entry into the nucleus is chaperoned by the dexamethasone-receptor complex [40]. Dexamethasone alone can also dilate the nuclear pore complex, so if cells are pretreated with dexamethasone, the nuclear pore complexes dilated and also allowed DNA carriers to enter the nucleus [34, 49]. In our experiments, because dexamethaone was loaded into the micelle core, it needed to diffuse out of the core in order to dilate the nuclear pore complex. If no enhancement of gene transfection was seen, then that would indicate that dexamethasone was not releasing from the micelles.

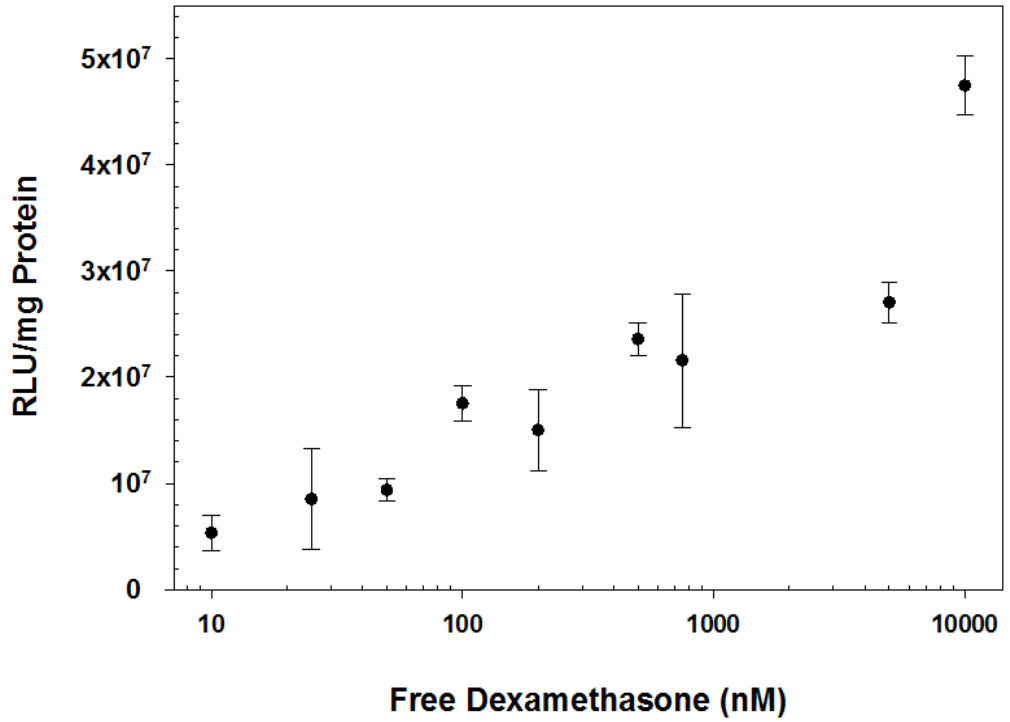

Figure 3.10: Transfection efficiency of reconstituted blank micelle/pDNA/bPEI$_{1.8kDa}$ complexes and free dexamethasone in MCF7 cells (5×10^5 cells at seeding). (n=4; mean ± SD)

To confirm that this transfection enhancement effect was directly due to the presence of dexamethasone, a transfection experiment was conducted where cells were treated with blank micelle/pDNA/bPEI$_{1.8kDa}$ complexes and free dexamethasone simultaneously and transfection levels were compared to blank micelle/pDNA/bPEI$_{1.8kDa}$ complexes only (no dexamethasone treatment). As shown in Figure 3.10, transfection efficiency increased with increasing dexamethasone dose, indicating that enhanced gene expression correlated directly with the presence of dexamethasone and confirms that dexamethasone alone was responsible for the increase in gene transfection, since all other conditions remained identical. Interestingly, the amount of dexamethasone required to enhance transfection was greater when cells were treated with free dexamethasone, and less dexamethasone was required to elicit an equivalent response when cells were treated with dex-loaded micelles. These results not only indicate that dexamethasone released from the core of the micelle and diffused out into the cytosol where it was able to act upon the nuclear pore complexes and enhance nuclear accumulation of the polyplexes, but also that the amount of dexamethasone necessary to enhance nuclear accumulation was reduced because of the delivery of dexamethasone was localized directly within the cell. This is important because dexamethasone can sometimes have a negative effect when administered in high doses, and by delivering the dexamethasone locally rather than systemically this can alleviate any potential unwanted side effects caused by interactions with excess dexamethasone. Finally, the toxicity of all three dex-loaded micelle/pDNA/bPEI$_{1.8kDa}$ complexes was significantly lower than “fresh” bPEI$_{25kDa}$/pDNA (N/P 5) complexes ($p < 0.04$) with cell

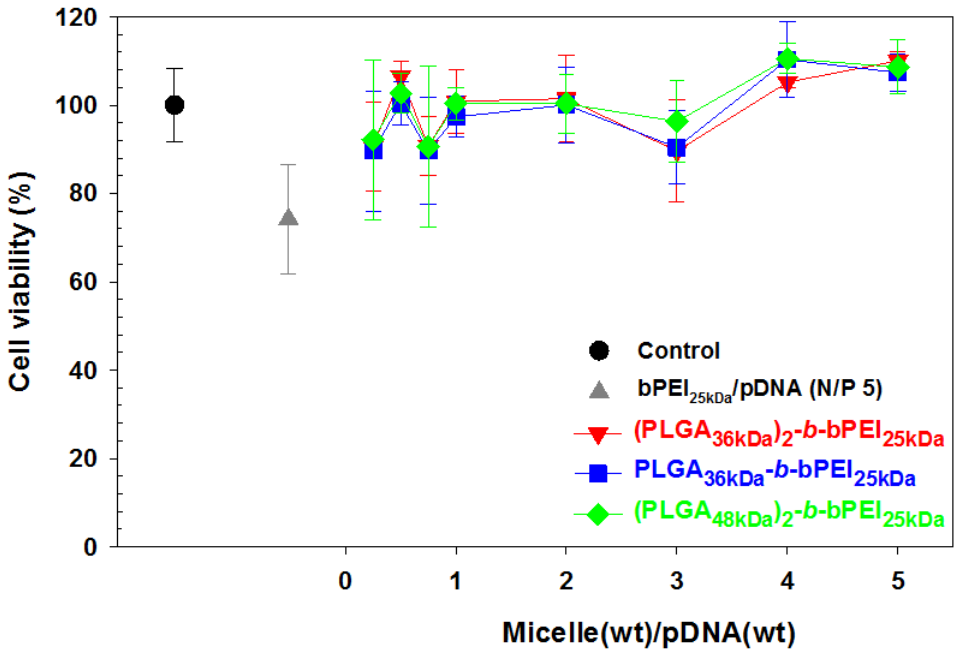

Figure 3.11: Cytotoxicity of reconstituted dex-micelle/pDNA/bPEI$_{1.8kDa}$ complexes (0.5 μg of pDNA) in MCF7 cells (2.5×10^5 cells at seeding). (n=4; mean ± SD)

viabilities greater than 90% at every condition tested (Fig. 3.11), including the condition that achieved 3.4-fold higher gene expression than "fresh" bPEI$_{25kDa}$/pDNA (N/P 5) complexes.

Based on these results, our system has many favorable characteristics that make it a good candidate for gene delivery: dex-micelle/pDNA/bPEI$_{1.8kDa}$ complexes can be lyophilized and reconstituted without the loss of physicochemical or biological characteristics, are relatively nontoxic, achieve equivalent transfection efficiency using less polymer compared to the blank carrier alone, and enhance gene transfection efficiency by increasing nuclear accumulation of the delivery vehicle. In addition, the possibility to customize the carrier even further exists due to the unique structure of

this particular copolymer. Conjugating targeting moieties to the carrier surface could increase cellular uptake in a particular population of interest, and coupled with the increased nuclear accumulation already afforded from dexamethasone, this creates a multifunctional dual-agent delivery system that can be lyophilized for storage and easily reconstituted for use without losing potency.

3.5. Conclusions

The intent of this study was to create a charged polymeric micelle-based gene delivery system that showed enhanced nuclear delivery and increased gene expression. Using $(PLGA)_n$-*b*-$bPEI_{25kDa}$ block copolymers to create micelles loaded with dexamethasone in the core, we were able to create a stable micelle system that retained its physicochemical characteristics following reconstitution and successfully transfected breast cancer cells with a reporter gene to achieve relatively high gene expression levels with minimal cytotoxicity. Transfection efficiency was enhanced by the presence of dexamethasone in the micelles, which increased nuclear accumulation of the polyplexes and subsequent gene expression. Finally, this system could be useful in reducing carrier-induced toxicity by decreasing the amount of polymeric carrier necessary to achieve equivalent gene transfection since dexamethasone was able to increase nuclear accumulation of the polyplexes. The results from this system are encouraging in the quest to develop nonviral carrier systems for gene therapy applications in humans.

3.6. Acknowledgements

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3.7. References

[1] Culver K. Gene Therapy - A Handbook for Physicians. New York: Mary Ann Liebert, Inc., 1994.

[2] Goncalves MA. A concise peer into the background, initial thoughts and practices of human gene therapy. Bioessays 2005;27(5):506-517.

[3] Cotrim AP, Baum BJ. Gene therapy: some history, applications, problems, and prospects. Toxicol Pathol 2008;36(1):97-103.

[4] Jolly DJ, Okayama H, Berg P, Esty AC, Filpula D, Bohlen P, et al. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyl transferase. Proc Natl Acad Sci USA 1983;80(2):477-481.

[5] Miller AD, Jolly DJ, Friedmann T, Verma IM. A transmissible retrovirus expressing human hypoxanthine phosphoribosyltrasnferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. Proc Natl Acad Sci USA 1983;80(15):4709-4713.

[6] Willis RC, Jolly DJ, Miller AD, Plent MM, Esty AC, Anderson PJ, et al. Partial phenotypic correction of human Lesch-Nyhan (hypoxanthine-guanine phosphoribosyltransferase-deficient) lymphoblasts with a transmissible retroviral vector. J Biol Chem 1984;259(12):7842-7849.

[7] Sangro B, Mazzolini G, Ruiz M, Ruiz J, Quiroga J, Herrero I, et al. A phase I clinical trial of thymidine kinase-based gene therapy in advanced hepatocellular carcinoma. Cancer Gene Ther 2010;17(12):837-843.

[8] Diaz-Nido J. NLX-P101, an adeno-associated virus gene therapy encoding glutamic acid decarboxylase, for the potential treatment of Parkinson's disease. Curr Opin Investig Drugs 2010;11(7):813-822.

[9] McPhee SW, Janson CG, Li C, Samulski RJ, Camp AS, Francis J, et al. Immune responses to AAV in a phase I study for Canavan disease. J Gene Med 2006;8(5):577-588.

[10] Leone P, Janson CG, Bilaniuk L, Wang Z, Sorgi F, Huang L, et al. Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. Ann Neurol 2000;48(1):27-38.

[11] Romano G, Marino I, Pentimalli F, Adamo V, Giordano A. Insertional mutagenesis and development of malignancies induced by integrating gene delivery systems: implications for

the design of safer gene-based interventions in patients. Drug News Perspect 2009;22(4):185-196.

[12] Kang HC, Lee M, Bae YH. Polymeric gene carriers. Crit Rev Eukaryot Gene Expr 2005;15(4):317-342.

[13] Deyle D, Russell D. Adeno-associated virus vector integration. Curr Opin Mol Ther 2009;11(4):442-447.

[14] Kay MA. AAV vectors and tumorigenicity. Nat Biotechnol 2007 Oct;25(10):1111-1113.

[15] Beer S, Bellovin DI, Lee JS, Komatsubara K, Wang LS, Koh H, et al. Low-level shRNA cytotoxicity can contribute to MYC-induced hepatocellular carcinoma in adult mice. Mol Ther 2009;18(1):161-170.

[16] Bosticardo M, Ghosh A, Du Y, Jenkins NA, Copeland NG, Candotti F. Self-inactivating retroviral vector-mediated gene transfer induces oncogene activation and immortalization of primary murine bone marrow cells. Mol Ther 2009;17(11):1910-1918.

[17] Ferrer-Miralles N, Vazquez E, Villaverde A. Membrane-active peptides for non-viral gene therapy: making the safest easier. Trends Biotechnol 2008 May;26(5):267-275.

[18] Kang HC, Bae YH. pH-Tunable endosomolytic oligomers for enhanced nucleic acid delivery. Adv Funct Mater 2007;17(8):1263-1272.

[19] Meng F, Hennink WE, Zhong Z. Reduction-sensitive polymers and bioconjugates for biomedical applications. Biomaterials 2009 Feb 4;30(12):2180-2198.

[20] Kim YH, Park JH, Lee M, Kim YH, Park TG, Kim SW. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J Control Release 2005 Mar 2;103(1):209-219.

[21] Kang HC, Kang H-J, Bae YH. A reducible polycationic gene vector derived from thiolated low molecular weight branched polyethyleneimine linked by 2-iminothiolane. Biomaterials 2011;32(4):1193-1203.

[22] Ogris M, Wagner E. Tumor-targeted gene transfer with DNA polyplexes. Somat Cell Mol Genet 2002;27(1-6):85-95.

[23] Kang HC, Kim S, Lee M, Bae YH. Polymeric gene carrier for insulin secreting cells: Poly(l-lysine)-g-sulfonylurea for receptor mediated transfection. J Control Release 2005 May 7;105:164-176.

[24] Chan CK, Jans DA. Using nuclear targeting signals to enhance non-viral gene transfer. Immunol Cell Biol 2002;80:119-130.

[25] Pouton CW, Wagstaff KM, Roth DM, Moseley GW, Jans DA. Targeted delivery to the nucleus. Adv Drug Deliv Rev 2007 Aug 10;59(8):698-717.

[26] Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev 2001;47(1):113-131.

[27] Qiu L, Bae YH. Self-assembled polyethylenimine-graft-poly(e-caprolactone) micelles as potential dual carriers of genes and anticancer drugs. Biomaterials 2007;28(28):4132-4142.

[28] Mishra D, Kang HC, Bae YH. Reconstitutable charged polymeric (PLGA)2-b-PEI micelles for gene therapeutics delivery. Biomaterials 2011;32(15):3845-3854.

[29] Davis ME, Chen Z, Shin D. Nanoparticle therapeutics: an emerging treatment modality for cancer. Nat Rev Drug Discov 2008;7(9):771-782.

[30] Kim D, Lee ES, Oh KT, Gao ZG, Bae YH. Doxorubicin-loaded polymeric micelles overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. Small 2008;4(11):2043-2050.

[31] Jeong YI, Seo SJ, Park IK, Lee HC, Kang IC, Akaike T, et al. Cellular recognition of paclitaxel-loaded polymeric nanoparticles composed of poly(gamma-benzyl L-glutamate) and poly(ethylene glycol) diblock copolymer endcapped with galactose moiety. Int J Pharm 2005;296(1-2):151-161.

[32] Louise C. Nonviral vectors. Methods Mol Biol 2006;333:201-226.

[33] Alber F, Dokudovskaya S, Veenhoff LM, Zhang W, Kipper J, Devos D, et al. The molecular architecture of the nuclear pore complex. Nature 2007;450(7170):695-701.

[34] Kastrup L, Oberleithner H, Ludwig Y, Schafer C, Shahin V. Nuclear envelope barrier leak induced by dexamethasone. J Cell Physiol 2006;206(2):428-434.

[35] Strambio-De-Castillia C, Niepel M, Rout MP. The nuclear pore complex: bridging nuclear transport and gene regulation. Nat Rev Mol Cell Biol 2010;11(7):490-501.

[36] Kramer A, Ludwig Y, Shahin V, Oberleithner H. A pathway separate from the central channel through the nuclear pore complex for inorganic ions and small macromolecules. J Biol Chem 2007;282(43):31437-31443.

[37] Lam AP, Dean DA. Progress and prospects: nuclear import of nonviral vectors. Gene Ther 2010;17(4):439-447.

[38] Bae YM, Choi H, Lee S, Kang SH, Kim YT, Nam K, et al. Dexamethasone-conjugated low molecular weight polyethylenimine as a nucleus-targeting lipopolymer gene carrier. Bioconjug Chem 2007;18(6):2029-2036.

[39] Kim H, Kim HA, Bae YM, Choi JS, Lee M. Dexamethasone-conjugated polyethylenimine as an efficient gene carrier with an anti-apoptotic effect to cardiomyocytes. J Gene Med 2009;11(6):515-522.

[40] Rebuffat A, Bernasconi A, Ceppi M, Wehrli H, Verca SB, Ibrahim M, et al. Selective enhancement of gene transfer by steroid-mediated gene delivery. Nature Biotech 2001;19(12):1155-1161.

[41] Rebuffat A, Nawrocki AR, Nielsen PE, Bernasconi A, Bernal-Mendez E, Frey BM, et al. Gene delivery by a steroid-peptide nucleic acid conjugate. FASEB J 2002;16(11):1426-1428.

[42] Ou M, Xu R, Kim SH, Bull DA, Kim SW. A family of bioreducible poly(disulfide amine)s for gene delivery. Biomaterials 2009;30(29):5804-5814.

[43] Kang HC, Samsonova O, Bae YH. Trafficking microenvironmental pHs of polycationic gene vectors in drug-sensitive and multidrug-resistant MCF7 breast cancer cells. Biomaterials 2010;31(11):3071-3078.

[44] Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV. Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. J Control Release 2007;119(1):77-85.

[45] Lee W-k, Park J-y, Yang EH, Suh H, Kim SH, Chung DS, et al. Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer. J Control Release 2002;84(3):115-123.

[46] Park TG. Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition. Biomaterials 1995;16(15):1123-1130.

[47] Braunecker J, Baba M, Milroy GE, Cameron RE. The effects of molecular weight and porosity on the degradation and drug release from polyglycolide. Int J Pharm 2004;282(1-2):19-34.

[48] Jalil R, Nixon JR. Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. J Microencapsul 1990;7(3):297-325.

[49] Shahin V, Albermann L, Schillers H, Kastrup L, Schafer C, Ludwig Y, et al. Steroids dilate nuclear pores imaged with atomic force microscopy. J Cell Physiol 2005;202(2):591-601.

CHAPTER 4

SYNOPSIS OF RESULTS, CONCLUSIONS, AND FUTURE DIRECTIONS

4.1. Synopsis of Results and Conclusions

Current nonviral based gene delivery vehicles are plagued by low gene transfection and gene expression rates, and many formulations are not well-suited for use in a clinical setting. The purpose of this research was three-fold: to create a polymeric micelle-based delivery system that could be co-loaded with two different therapeutic molecules, to demonstrate that these micelles system could also be used for gene therapeutics delivery and would retain efficacy following lyophilization and reconstitution, and finally that the co-loaded micelle system could preferentially accumulate in the cell nucleus, thereby increasing gene transfection and subsequently gene expression.

A cationic copolymer synthesized by conjugating poly(lactide-co-glycolide) (PLGA) and branched polyethylenimine (bPEI) was used to form charged polymeric micelles with a core-shell architecture which were investigated for nonviral gene therapeutics delivery. The copolymer was designed to utilize the proton-buffering ability and high transfection efficiency of bPEI combined with decreased overall cytotoxicity and the ability to load hydrophobic drugs in the micelle core conferred by the PLGA

block. The resulting micelles retained their physicochemical characteristics following lyophilization and reconstitution, and the $bPEI_{25kDa}$ block retained about 65% of its proton buffering capacity following copolymerization.

Micelle/pDNA complexes were also evaluated for changes in their physicochemical and biological characteristics of following lyophilization and reconstitution. Polyplexes retained their particle size and zeta potential following reconstitution. Adding a small amount of $bPEI_{1.8kDa}$ completely shielded pDNA in the complexes and transfection efficiency was enhanced 50-100 fold without affecting complex size. Reconstituted micelle/pDNA/$bPEI_{1.8kDa}$ WR1 complexes achieved 16-fold higher transfection rates compared to their fresh counterparts, and although transfection levels were slightly lower (3.9-fold) than control fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes, transfection levels were nearly 40-fold higher than reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. Finally, the micelle/pDNA/$bPEI_{1.8kDa}$ system had very low toxicity even with pDNA doses up to 20µg, and transfection levels increased linearly with increasing pDNA dose. This study concluded that this PLGA-*b*-bPEI micelle-based system was well suited as a reconstitutable gene delivery system and could be useful as a delivery system in gene therapy applications in the future.

Having confirmed that this copolymer architecture and the resulting micelles were reconstitutable and suitable for gene therapeutics delivery, the next step was to determine whether the system could be co-loaded with two different therapeutic molecules and if this system could be used to enhance nuclear accumulation of the gene carrier. Several different copolymers having a PLGA-*b*-bPEI architecture were

synthesized, characterized, and used to make polymeric micelles: $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ diblock copolymer, $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ triblock copolymer, and $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ triblock copolymer. Micelles were loaded with dexamethasone during formation, and the resulting dex-loaded micelles were characterized in terms of particle size, zeta potential, dexamethasone content, proton buffering capacity, and reconstitutability. Dex-loaded micelles were then complexed with pDNA and evaluated for particle size, zeta potential, pDNA condensation ability, and cell transfection efficiency.

As expected, all three dex-loaded micelle systems retained their particle size and zeta potential characteristics following reconstitution, forming micelles that were 70-100nm in diameter and strongly positive zeta potential (35-45mV). Proton buffering capacity was also retained in all three copolymer systems although buffering capacity decreased following copolymerization (ranging from 32% to 72% compared to free $bPEI_{25kDa}$ alone), and was affected by both the molecular weight and block ratio of PLGA contained in the copolymer; proton buffering capacity decreased as the number of PLGA blocks increased, and as the molecular weight of the PLGA block increased. The amount of dexamethasone incorporated into the micelle core varied from 7.6 wt% to 17.4 wt% as determined by UV spectroscopy. The dex-loaded micelle systems had very low cytotoxicity overall and toxicity was unaffected by the presence of dexamethasone.

Dex-loaded micelles were complexed with pDNA and then mixed with $bPEI_{1.8kDa}$ to form dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes and characterized for particle size, zeta potential, pDNA condensation, transfection efficiency, and cytotoxicity. Polyplexes also

retained their particle size and zeta potential following reconstitution, and reconstituted complexes were used for all further experimentation. Transfection efficiencies using dex-micelle/pDNA/$bPEI_{1.8kDa}$ complexes were compared to blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes, fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes and reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. Transfection levels ranged from only 1.4-fold to 4.8-fold lower than fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes, even for polyplexes with WR < 1. In fact, using $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ dex-micelle/pDNA/$bPEI_{1.8kDa}$ WR 3 complexes transfection levels were 3.3-fold higher than fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes. In contrast, transfection levels using blank micelle/pDNA/$bPEI_{1.8kDa}$ WR 1 complexes were between 1.4-fold up to 8.1-fold lower than fresh $bPEI_{25kDa}$/pDNA (N/P 5) complexes. Thus loading dexamethasone into the micelle core reduced the amount of polymer necessary to achieve equivalent transfection levels by up to 4-fold.

The benefits stemming from the presence of dexamethasone were apparent after comparing transfection levels achieved using dex-loaded micelle complexes and blank micelle complexes, and even more so after comparing transfection levels from dex-loaded micelle complexes to reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. Dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ systems achieved expression levels ranging from 1.1-fold up to 35-fold higher than their counterpart blank micelle/pDNA/$bPEI_{1.8kDa}$ complexes. All three copolymer systems showed marked increase in gene transfection using dex-micelle/pDNA/$bPEI_{1.8kDa}$ WR < 1 complexes compared to blank micelle/pDNA/$bPEI_{1.8kDa}$ WR < 1 complexes: 10.7-fold to 21.9-fold higher transfection levels for $PLGA_{36kDa}$-*b*-$bPEI_{25kDa}$ /pDNA/$bPEI_{1.8kDa}$ complexes ($p < 0.007$), 19.6-fold to

32.7-fold higher transfection levels for $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ complexes ($p < 0.0006$), and 20.8-fold to 35-fold higher transfection levels for $(PLGA_{48kDa})_2$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ complexes ($p < 0.008$), indicating that the presence of dexamethasone was beneficial in enhancing transfection levels. This could be very useful as a method to decrease the amount of polymer carrier necessary to achieve equivalent gene expression; decreasing the amount of polymer administered would also diminish unwanted toxicity resulting from the carrier, making these dex-loaded micelle carriers a favorable candidate for enhanced gene delivery to the nucleus.

The most exciting results were obtained in comparing expression levels using dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ systems to reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. All three dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ WR 0.25 complexes had expression levels 30- to 44-fold higher than reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes, and increased up to 100-fold higher using dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ WR 1 complexes. Previously we had shown that blank $(PLGA_{36kDa})_2$-*b*-$bPEI_{25kDa}$ micelle/pDNA/$bPEI_{1.8kDa}$ WR 1 complexes were able to achieve transfection levels 39-fold higher than reconstituted $bPEI_{25kDa}$/pDNA (N/P 5) complexes. Thus the presence of dexamethasone increased expression levels over 60-fold higher compared to the blank system, clearly establishing that nuclear accumulation of polyplexes increased with the presence of dexamethasone in the micelle core. Finally, the toxicity of the dex-loaded micelle/pDNA/$bPEI_{1.8kDa}$ complexes was significantly lower than “fresh” $bPEI_{25kDa}$/pDNA (N/P 5) complexes, with cell viabilities greater than 90% at

every condition tested, including the condition that achieved 3.4-fold higher gene expression than "fresh" $bPEI_{25kDa}$/pDNA (N/P 5) complexes.

In conclusion, cationic PLGA-*b*-bPEI copolymers were synthesized and used to create charged polymeric micelles for targeted nuclear delivery of gene therapeutics. This system demonstrated several advantages over current polymer carriers due to their reconstitutable nature and enhanced nuclear accumulation, leading to increased gene expression while decreasing carrier requirements. These PLGA-*b*-bPEI copolymers were significantly less toxic than traditional $bPEI_{25kDa}$ control while achieving nearly equivalent or higher expression of a reporter gene in vitro. The advantage of this particular micelle-based system is that the micelle architecture can be utilized for the co-delivery of two different therapeutics simultaneously and shows enhanced nuclear accumulation and gene transfection which is particularly important for gene therapy applications. The results presented using this system demonstrate positive progress towards improving nonviral polymeric carriers as effective delivery systems for gene therapy.

4.2. Future Directions

Initial characterization of the PLGA-*b*-bPEI copolymer system indicated that this cationic micelle system shows promise as a nonviral vector for gene delivery applications. However, further optimization and experimentation remain in order to determine the full therapeutic potential of this carrier system. Having thoroughly tested this system using a reporter gene, the next phase should include delivery and evaluation of gene expression using therapeutically relevant genes. Optimizing the block copolymer architecture in terms of block ratio and block molecular weight to determine maximum

drug carrying capacity will influence the suitability of this system for treating different disease models. Examining carrier behavior and transfection efficiency in different cell types, modifying the carrier to selectively accumulate in other target populations, investigating whether reconstitution characteristics extend to protect more sensitive drug cargo such as protein drugs all would serve to elucidate the potential use of this micelle system for gene therapy applications.

4.2.1. Combination Therapy to Treat Inflammatory Diseases

Glucocorticoid receptors are ubiquitous to nearly every cell type in the body. They are usually located in the cytoplasm and upon binding to glucocorticoid hormones the ligand-receptor complex will translocate into the nucleus through channels formed by nuclear pore complexes. The glucocoricoid receptor can bind directly to DNA to control gene transcription, or it can bind other proteins to signal gene transcription. Glucocorticoids are involved in the regulation of carbohydrate, protein and fat metabolism [1], modulation of immune responses by suppressing chemokine and cytokine production [2, 3], and serve important roles in the central nervous system [4, 5], digestive system [6], renal system [7], hematopoetic system [8] and the reproductive system [9].

Dexamethasone is a synthetic steroid hormone that can bind to the glucocorticoid receptor in the cytosol to form a receptor-ligand complex. This complex can serve as a transcription factor by binding to the promoter region of a gene to stimulate gene expression. It has been shown to have enhancing and suppressing effects

on transgene expression in vitro, indicating that this molecule could be important to achieving successful gene therapy [10].

Dexamethasone can also act as an anti-inflammatory and an immunosuppressant, and these properties have made it useful in the clinic as a secondary treatment to alleviate some of the side effects resulting from chemotherapy by suppressing cytokine production. By utilizing both these features, our dex-loaded micelle system could be used to deliver both dexamethasone and DNA/RNA simultaneously. This combination therapy approach could benefit people suffering from diseases that already require and respond to anti-inflammatory treatment such as asthmatic patients; delivering dexamethasone at the site of interest would increase the local concentration and allow better control of symptoms, and also decrease the possibility of developing glucocorticoid resistance which is common in patients requiring chronic glucocorticoid treatment. The efficacy of dex-loaded micelles could be evaluated using cell models that mimic conditions in asthmatic patients to determine whether lower concentrations of dexamethasone (as present in the micelles) would alleviate symptoms to the same degree, and whether the co-delivery of DNA could transfect the cells with genes that would also have an anti-inflammatory effect for stronger treatment. This technology could then be extended further to encompass other diseases that already use dexamethasone treatment.

4.2.2. *In Vivo* Efficacy Studies Using Dexamethasone-Loaded Micelles

Our characterization of the charged catinonic polymer-based dexamethasone-loaded micelle system for gene delivery was conducted *in vitro* using a cancer cell

model. Given the encouraging results in transfection enhancement due to the presence of dexamethasone, the next logical step would be to further evaluate this system *in vivo* which would enable us to determine whether the beneficial effects seen during the *in vitro* characterization are retained in a more realistic model environment. Because anti-inflammatory treatment is often administered to cancer patients, it would be of value to evaluate whether there is any anti-inflammatory therapeutic benefit from the dexamethasone in addition to its current role for transfection enhancement. Using DNA that codes therapeutically relevant genes such as anti-inflammatory genes would provide an additional layer of therapeutic benefit to the patient, creating a combinatorial anti-inflammatory treatment option which could be more effective than the current single-treatment approach of systemically administering anti-inflammatory drugs.

4.2.3 Delivery of siRNA

Ongoing research is increasingly discovering that many disease conditions are caused by changes in protein expression levels in the body. This can be due to increased expression, decreased expression, or the expression of mutant/misformed proteins. Altering protein expression by interfering with gene translation using RNA has become an active area of interest for altering disease progression. The delivery of small interfering RNA molecules (siRNA) against particular genes has been shown to be quite effective at reversing some disease states. Due to the nature of our micelle-based delivery system, instead of DNA, RNA molecules could easily be loaded by complexation

and delivered to the cells of interest with higher accumulation, which could result in more effective silencing because of increased interference in gene expression.

4.3. References

[1] Qi D, Pulinilkunnil T, An D, Ghosh S, Abrahani A, Pospisilik JA, et al. Single-dose dexamethasone induces whole-body insulin resistance and alters both cardiac fatty acid and carbohydrate metabolism. Diabetes 2004;53(7):1790-1797.

[2] Blaser C, Wittwer M, Grandgirard D, Leib SL. Adjunctive dexamethasone affects the expression of genes related to inflammation, neurogenesis and apoptosis in infant rat pneumococcal meningitis. PLoS ONE 2011;6(3):e17840.

[3] Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol 2010;120(2-3):69-75.

[4] McEwen BS, Kalia M. The role of corticosteroids and stress in chronic pain conditions. Metabolism 2010;59 Suppl 1:S9-15.

[5] Rose AK, Shaw SG, Prendergast MA, Little HJ. The importance of glucocorticoids in alcohol dependence and neurotoxicity. Alcohol Clin Exp Res 2010;34(12):2011-2018.

[6] Rose AJ, Vegiopoulos A, Herzig S. Role of glucocorticoids and glucocorticoid receptor in metabolism: insights from genetic manipulations. J Steroid Biochem Mol Biol 2010;122(1-3):10-20.

[7] Mesquita FF, Gontijo JA, Boer PA. Maternal undernutrition and the offspring kidney: from fetal to adult life. Braz J Med Biol Res 2010;43(11):1010-1018.

[8] Amsterdam M, Sasson R. The anti-inflammatory action of glucocorticoids is mediated by cell type specific regulation of apoptosis. Mol Cell Endrocrinol 2002;189(1-2):1-9.

[9] Barrell GK. Immunological influences on reproductive neuroendocrinology. Soc Reprod Fertil Suppl 2007;64:109-122.

[10] Akahane M, Kuriyama S, Ohgushi H, Akahane T, Kawamura K, Watanabe S, et al. Enhancing and suppressing effects of dexamethasone on transgene expression in vitro. Int J Mol Med 2002;10(1):107-112.

APPENDIX

INCREASING INSULIN SECRETION IN ENCAPSULATED ISLETS USING INSULIN STIMULATING PEPTIDES

A.1. Abstract

Donor scarcity is one problem affecting islet transplantation, due to huge islet requirements for patient insulin-independence (>10,000 islets/kg body weight). Increasing insulin output from pancreatic islets in response to elevated glucose levels using peptide stimulants such as Exendin-4 could decrease the number of islets required to achieve normoglycaemia. Exendin-4 was conjugated to poly(vinyl pyrrolidone-co-acrylic acid) using a PEG_{3400} spacer (VAPE). Conjugation was confirmed using RP-HPLC. Exendin-4 content in VAPE was determined to be 30 wt% using UV spectroscopy. Following a glucose challenge test, insulin secretion from the islets was quantified using radioimmunoassay techniques. When glucose levels were changed from 2.6mM (basal level) to 16.8mM (elevated level), insulin secretion increased 300% ($p=0.00009$) when VAPE stimulant was present compared to the control (no stimulant). Insulin secretion levels were not amplified at lower glucose levels (2.6mM-5.8mM) ($p=0.87$), the stimulatory effect was seen only at higher glucose levels (16.8mM). Insulin secretion levels were comparable when islets were stimulated with either Exendin-4 peptide or Exendin-4/polymer conjugate ($p=0.93$) using equivalent concentrations ranging from

1nM to 1uM, indicating there was not a dramatic loss of bioactivity caused by conjugation. When islets were encapsulated with Exendin-4/polymer conjugate, islets maintained steady levels of insulin secretion in response to cyclic changes in glucose concentrations over two weeks, indicating continued islet functionality and responsiveness over time. This Exendin-4/polymer conjugate could be useful in decreasing the number of islets required for transplantation by increasing insulin output from pancreatic islets in response to elevated glucose levels.

A.2. Introduction

Diabetes Mellitus is a disease which occurs when the body is no longer able to properly produce or utilize insulin. Although the actual cause of diabetes is still not properly understood, it is believed that disease development is influenced both by environmental factors and genetics. There are two major classes of diabetes mellitus, Type I and Type II, as well as gestational diabetes and prediabetes. Type I patients account for approximately 10% of the diabetic population, while Type II patients account for the other 90%. According to the Center for Disease Control, diabetes was listed as the sixth leading cause of death in 2002 [1] . There are approximately 21 million people with diabetes in the United States, and an estimated 177 million diabetic patients worldwide [2]. In 2005, 1.5 million new cases of diabetes were diagnosed. Annual costs per patient range from $1,000 to $15,000, and in 2002 it was reported that total diabetes-related expenditures amounted to $132 billion. It is estimated that by the year 2025, there will be more than 300 million people worldwide suffering from diabetes [1-4].

Type II diabetic patients retain the ability to produce insulin, and can often control their disease state through diet and exercise. However, patients suffering from Type I diabetes have lost the ability to produce insulin because their beta cells have been destroyed. Although there are several different treatment options available to Type I patients, the most commonly prescribed treatment is exogenous insulin administration. Thus, they must rely on repetitive insulin injections or infusions to control their blood sugar, and the success of this treatment is dependent upon patient compliance.

Current treatment options for this group are limited to whole pancreas or islet transplantation, administration of exogenous insulin, mechanical artificial pancreas transplant, and bioartificial pancreas transplant. Pancreas transplants are limited as a treatment option because of the scarcity of donor organs. Administering exogenous insulin is currently the most common treatment option, although it relies very heavily on patient compliance in order to be effective. In an effort to improve the quality of life for Type I patients, there has been a major effort to create a 'bioartificial pancreas' system to replace the lost insulin-producing function of the islets, thereby eliminating the need for multiple daily insulin injections. Research groups have investigated the use of systems made from hollow-fiber membranes [4, 5], genetically modified/altered islets or naked islets [6-9], stem-cells [10, 11], and encapsulation systems [12-15]. Extensive research has been done using encapsulation systems based on alginate and/or poly-L-lysine membranes, showing capsule stability and islet functionality for years [15-19]. Microencapsulation systems have several beneficial features including islet

immunoprotection through a semipermeable membrane [20], smaller implant volume [21], and easily accessible implantation sites [21-23]. For these reasons, a microencapsulation system was chosen for this research application. Islets were encapsulated in a five-layer membrane double-wall design consisting of alternating layers of alginate and poly-L-lysine, shown as a schematic in Figure A-1.

Current guidelines suggest that 15,000 islets or islet equivalents should be implanted per kilogram of body weight [24-26]. There is a shortage of readily available islets due to the scarcity of pancreas donors, so any methods that decrease the number of islets required to achieve and maintain normoglycemia would be advantageous. One method of achieving this is by using bioactive molecules capable of stimulating insulin

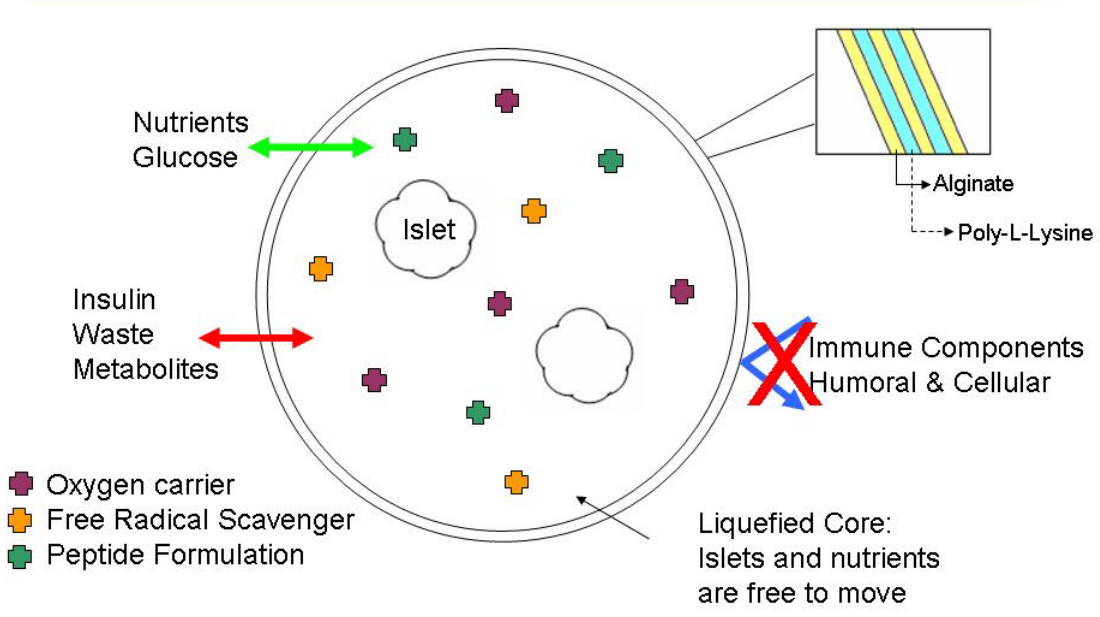

Figure A-1: Schematic of encapsulated islets

secretion from the islets. One candidate molecule is glucagon-like peptide 1 (GLP-1), a small hormone which signals the production of insulin in islets in response to high blood glucose levels, and exendin-4, a GLP-1 analog. GLP-1 is one of the products produced from the preproglucagon gene, synthesized mainly by enteroendocrine L cells of the intestine [27-30]. It is a small peptide with a molecular weight of 3348 Daltons [29] whose most important action is the stimulation of insulin production in response to glucose. This molecule in particular responds to very high glucose levels in the body, greater than 3mM glucose levels [31], which is why it is ideal for treating Type I diabetes patients. Other molecules such as sulfonylureas respond at lower glucose levels, making them less ideal for treating Type I diabetics [32]. Fasting glucose levels for Type I diabetes patients are typically within that range, causing constant stimulation from the sulfonylureas, thus these molecules are typically used for treating Type 2 diabetes. Some other actions of GLP-1 include inhibition of gastric emptying and glucagon secretion, control over feelings of satiety and hunger, several glucose lowering actions, and even the regulation of islet regeneration and islet neogenesis [33-36].

Another candidate molecule is Exendin-4, a small peptide isolated from the saliva of the Gila Monster *Helodermas suspectum*. The pancreatic activity of the monster venom was first discovered in 1982 [37], and sequencing and purification yielded two molecules: exendin-4 and helodermin [38, 39]. Exendin-4 contains 53% homology with mammalian GLP-1, shows high affinity binding to the mammalian GLP-1 receptor, and nearly equal potency and efficacy in stimulating cAMP production compared to mammalian GLP-1 [40-42]. In addition, it is more resistant to enzymatic

degradation in the body, resulting in a longer plasma circulation half-life. However, it also has a low molecular weight, 4187Da [38], meaning it too would rapidly diffuse out of the capsules and become lost into systemic circulation. Because of its increased potency over GLP-1, it was modified to create a formulation which would increase its retention within the microcapsules, and provide a depot of peptide to the islets.

A.2.1. Type I Diabetes Mellitus

Type 1 Diabetes Mellitus, formerly known as 'juvenile diabetes' or 'insulin-dependent diabetes mellitus (IDDM)', constitutes about 10% of the patients diagnosed with diabetes [43]. It is characterized by the systematic destruction of β-cells in the pancreas by the host's immune system, leading to a dependence on exogenous insulin sources to control blood glucose levels [44]. Patients typically have to check their blood glucose levels several times a day using a glucometer and then administer the appropriate amount of insulin into their system, usually via an injectable form.

A.2.1.1. Pathology of Type I Diabetes

Type I diabetes results from the destruction of β-cells in the pancreas by the immune system. The loss of these cells means the body can no longer produce insulin, resulting in elevated glucose levels in the blood. Over time, higher blood glucose levels lead to serious health complications such as diabetic neuropathy, diabetic retinopathy, kidney disease, diabetic ketoacidosis, cardiac problems (i.e., heart disease and stroke), and even death [45-50]. Disease progression can be retarded by rigorously controlling cholesterol, blood pressure, and blood lipids. Most importantly, blood sugar levels can

be monitored and maintained through the careful administration of insulin. However, this means that the patient must check their blood glucose levels through a finger prick and then self-administer the necessary amount of insulin through an injection several times a day. This process is very painful and inconvenient for the patient, and does not preclude the development of complications. In addition, miscalculation of insulin requirements by the patient can pose serious health risks.

A.2.1.2. Current Treatment Options

At the present time, there is no cure for Type I diabetes. However, several options exist to help patients manage their disease. One option is to have a whole organ transplant, replacing the diseased pancreas with a donor pancreas [51]. This is difficult due to the general scarcity of organs and cost ($125,800 + $6900 per followup visit [52]). If a patient receives an organ transplant, there is a high chance the organ could be rejected, or blood clots could develop within the pancreas and the tissue would die from necrosis. In addition, the patient's pancreas is usually completely functional except for insulin production, so with the addition of the new pancreas there is overproduction/overexpression of some enzymes in the body, which can lead to other problems such as urinary tract infections, hyperinsulinemia, metabolic acidosis, hematuria, and reflux pancreatitis [53-56].

The most common treatment is exogeneous insulin administration through multiple daily injections, an insulin pump, or using an inhalable insulin form. Patients need to monitor their blood sugar levels several times a day using a glucose monitor and administer insulin accordingly. This procedure is quite painful, requiring needle sticks to

obtain blood for the glucose meter and injections to administer insulin. If a patient uses a continuous infusion insulin pump, they might still require booster insulin shots around mealtime. These methods of insulin administration are not perfect, and patients can easily misjudge the amount of insulin they require. This can cause any number of complications; if they administer too much insulin and cause their blood sugar to plunge, they can become disoriented and go into shock. If they administer too little, then their blood sugar will remain very high for too long, causing damage to sensitive tissues such as the eyes [57-59].

Another option is the implantation of an artificial pancreas, sometimes called a mechanical artificial pancreas. This device consists of three parts: a glucose sensor, an insulin pump, and a computer. The problem with this type of device is that a stable and accurate glucose sensor has yet to be developed, and an error in delivery or sensing could be quite risky, causing hypoglycemia or worse [60-64]. The mechanical pancreas also does not respond to changes in glucose as rapidly as islets, which can detect changes in less than 10 minutes [65]. These complications have led many researchers to look for an alternative solution to create a bioartificial organ to replace and mimic the natural system as closely as possible, improving overall quality of life and allowing these patients to resume a lifestyle similar to people without diabetes. Using a bioartificial system is beneficial because it replicates only the endocrine functions of the pancreas, which is the functionality lost when the islets of Langerhans are destroyed. This system provides the ability to monitor and sense changes in glucose levels rapidly. This treatment option also bypasses the need to administer immunosuppressive drugs to the

patient, thus avoiding potential side effects and complications associated with immunosuppression [66]. Various bioartificial pancreas systems currently under investigation are described in the next section, and summarized in Table A1.

A.2.2. Bioartificial Pancreas Systems

There are several artificial pancreas systems currently under development by various groups. Different approaches include implanting hollow fiber membranes filled with islets/cells [4-6], implanting microcapsules containing islets/cells within immunoprotective membranes [12-15], directly injecting naked islets [9, 10], implanting genetically modified islets [7, 8], and implanting stem cells [11, 12]. The intended implantation site impacts the type of approach used for the artificial system. Implanting cells or islets directly is usually done through the portal vein in the liver, where there is an adequate continuous supply of nutrients and direct access to the digestive system [24, 67]. Capsules or hollow fiber membrane systems are typically implanted in the peritoneal cavity [68-70]. Using an encapsulation system provides the advantage of immunoprotection of the islets through the capsule membrane. The molecular weight cutoff of the membrane can be controlled by manipulating the conditions of capsule manufacture so it is impermeable to the immunoglobulins and other molecules produced during an immune response [71]. It has been shown that implanted alginate-poly-L-lysine microcapsules are very well tolerated in the body [72]. In addition, the use of xenogenic islet sources within the encapsulation system did not require any immunosuppression regimens in the patients. The encapsulation membrane was enough of a barrier to prevent large scale immune responses [73].

Table A1: Types of Artificial Pancreas Systems

Artificial Pancreas System	Advantages	Disadvantages
Macrocapsule Systems such as Hollow Fiber Membranes [5, 6]	Provide local extracellular environment to support islets, anchors islets within a specific area to control insulin delivery	Hollow fiber membranes could become encapsulated by fibrous tissue, trigger immune responses, degradation products could cause secondary immune reactions
Genetically Altered Islets [7, 8]	Ability to fight apoptosis, perhaps stimulate local angiogenesis	Some risks when using viral vectors for transfection, and very low transfection efficiency using non-viral gene vectors
Naked Islets [9, 10]	Implanted into the portal vein, able to restore normoglycemia	Readily accessible to attack by the host immune system
Stem Cells [11, 12]	Can be stimulated to differentiate into islet-like cells which are able to produce insulin, produced from the patient's own cells	This process takes time, and is not always a viable option depending on the patient's health status
Microcapsule Systems such as alginate-PLL capsules [13-15]	Immunoisolated system, does not require immunosuppresion, allows the use of xenogenic cells, physically separates cells from the host immune system	Some biocompatibility issues if poly-L-lysine is used and there is incomplete complexation

Based on the literature, encapsulation systems comprised of alginate and poly-L-lysine have been used quite extensively, and are biocompatible in animals and humans for years [21]. For this reason, an alginate-poly-L-lysine microcapsule system having a five-layer membrane composed of alternating layers of alginate and poly-L-lysine was selected. The molecular weight of the poly-L-lysine dictates the molecular weight cutoff (MWCO) of the membrane. The cut-off value can be modified by several factors including varying the molecular weight of the poly-L-lysine or changing the incubation time [18]. The MWCO for this system is 100,000 Daltons, selected because this pore size is large enough to promote free exchange of oxygen, nutrients, and insulin but prevents large antibodies (150kDa – 900kDa [74]) from accessing the islets. Although it has been suggested that this MWCO may not be sufficient for protection [75], studies have shown that although there may be some permeation into alginate capsules *in vitro*, the capsules may be xenoprotective *in vivo* [76]. The core of the capsule will be liquefied so that the islets are mobile within the capsule, rather than being immobilized within a matrix. These conditions are similar to culture conditions *in vitro* in media without the capsule present. This system will protect the islets from the host immune system because the physical barrier of the membrane and its molecular weight cutoff prevent immune components from accessing the islets. The immuoprotection afforded by the membrane eliminates the need for immunosuppressive drug regimens, which create a number of complicating side-effects for the patient [77, 78].

A.2.3. Alginate/Poly-L-Lysine Microcapsules

Alginate has been shown to be relatively biocompatible [20, 21], and for this application an ultrapure low viscosity mannuronic acid alginate was used. Poly-L-lysine (PLL) affects the permeability of the membrane; changing the molecular weight of the PLL controls the molecular weight cutoff of the entire membrane. For this application, PLL with a molecular weight 10,000-20,000Da was selected to give a molecular weight cutoff around 100kDa for the capsule membrane. Goosen *et al.* [19] demonstrated that several properties of these microcapsules including shape, permeability, and size can be controlled. It has also been shown by Sun *et al.* [79] that transplanting islets immobilized in alginate-poly-L-lysine capsules is an effective way to treat diabetes. Double-wall membrane capsules are functionally superior to single-wall membrane capsules, forming fewer deformed capsules during microcapsule preparation [80]. Capsules implanted into the peritoneal cavity have been shown to be stable and yield viable islets upon retrieval several months/years after the initial implantation [81-86]. Using microcapsule systems allows the inclusion of cells from xenogenic sources, and due to their smaller size, implantation is easier in a murine diabetes model. For these reasons, the alginate/poly-L-lysine microcapsule system has been selected for this bioartificial pancreas application.

A.2.3.1. Alginate

Alginate is a naturally occurring polymer that exists in brown seaweeds and bacterium whose composition varies depending on the source of isolation [87, 88]. Alginates are unbranched binary copolymers with widely varying compositions and

structures. The two components of alginates are β–D-mannuronic acid (M) and α– L-guluronic acid (G). The composition and sequential structure of the alginate has a large effect on the stability, biocompatibility, and integrity of the alginate capsules formed [89-91]. It has been shown that when an outer coating of polycation such as Poly-L-lysine is applied to alginate microcapsules, intermediate-G or low-G alginates form more stable junctions than high-G alginates [89, 91]. The alginate selected for use in this application is an ultrapure low viscosity high mannuronic acid alginate, i.e., low-G content.

A.2.3.2. Poly-L-Lysine

In order to create a selectively porous membrane, polycations are often complexed to the alginate layers of microcapsules. The most commonly used polycation is poly-L-lysine, although other molecules have also been used including polymers of arginine, mixtures of lysine and arginine, and polyorthinine [21]. Poly-L-lysine is a highly positively charged amino acid chain, and is commonly used in encapsulation to control the molecular weight cutoff of the capsule membrane [85, 92, 93]. Because poly-L-lysine has been shown to elicit strong immune responses in the body, it is usually sandwiched between layers of alginate to improve biocompatibility of the final biomaterial [94].

A.2.4. Insulin Secretion

Insulin secretion is stimulated mainly by glucose, but can also be stimulated by the presence of free fatty acids or small peptides such as glucagon-like peptide-1. When glucose levels rise in the body, cells begin to metabolize the glucose. This alters the ratio

of ATP to ADP in the cell, leading to the closure of ATP-sensitive potassium channels and depolarization of the cell. Cell depolarization causes the opening of voltage-gated calcium channels, increasing cellular calcium levels. The calcium influx causes the activation of protein kinases, which triggers the release of insulin outside the cell through exocytosis. The activation of protein kinases can also be achieved by two other methods; the metabolism of free fatty acids into long chain fatty acyl moieties or the binding of molecules to the GLP-1 receptor. When molecules bind to the GLP-1 receptor on the cell surface cAMP levels in the cell are upregulated, leading to the activation of protein kinases and subsequent exocytosis of insulin [95]. These processes are diagrammed in Figure A-2.

A.2.5. Glucagon-like Peptide 1 (GLP-1)

Glucagon-like peptide 1 (GLP-1) is a 30 amino acid peptide derived from proglucagon [96]. Its secretion from L-cells in the intestine is stimulated by intraluminal glucose levels [29, 34, 97]. The biologically active molecule is GLP-1(7-36) amide, which is formed by cleavage and amidation of GLP-1(PG(78-107)) [98]. The molecular weight of the peptide is 3348Da, its plasma half-life is about 5 minutes, and its metabolic clearance rate is about 12-13 minutes [29, 34]. In the body GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase IV, becoming the biologically inactive GLP-1(9-36 amide) following the cleavage of its two N-terminal amino acid residues [29].

GLP-1 functions after binding to its cell surface receptor. GLP-1 receptors are highly expressed on the β-cell surface [29] and in the lung, with lower amounts

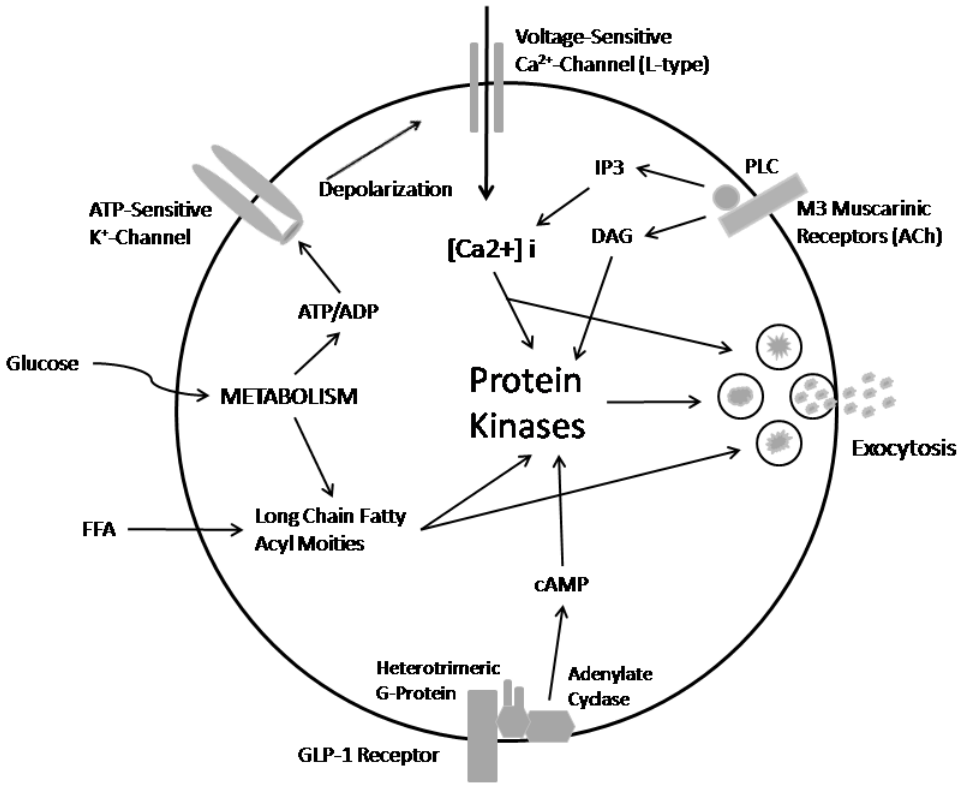

Figure A-2: Causes of insulin secretion from cells (adapted from ref [95])

detectable in the brain, liver, skeletal muscle, adipose tissue, and kidney [98]. GLP-1 stimulates the production of insulin after binding to its receptor, triggering the formation of cAMP [99-101]. This leads to a sequence of events involving the phosphorylation of proteins and the activation of cAMP-dependent protein kinase, which control insulin secretion [99].

In addition to stimulating glucose-sensitive insulin production, GLP-1 is known to decrease/inhibit glucagon production [29, 34, 102-104], inhibit gastric motility [35, 36], and control feelings of satiety/hunger by inhibiting food intake [105-108]. Recent

studies also indicate that GLP-1 may indirectly promote the expansion of the β–cell mass [109-112]. Of particular interest is GLP-1's ability to stimulate the production of insulin in response to elevated glucose levels. This peptide is only active after the ingestion of a meal, when glucose levels rise very rapidly. This mechanism will boost the amount of insulin secreted by islets in addition to what they would normally produce in response to rising glucose levels. In addition, it may provide some benefit in maintaining islet health over time by promoting β-cell regeneration.

A.2.6. Exendin-4

Exendin-4 is a small 39-amino acid peptide (4187Da) isolated from the saliva of the Gila Monster *Helodermas suspectum* [38]. The venom from this lizard was noted to have pancreatic secretagogue effects in 1982 [37]. Through sequencing and purification of the venom, two peptides were discovered to be responsible for this phenomenon – helodermin and exendin-4. Exendin-4 contains 53% homology with mammalian GLP-1 (Figure A-3) and has been shown to bind with very high affinity to the GLP-1 receptor. It is also capable of stimulating cAMP production with equivalent efficacy and potency compared to mammalian GLP-1 [40]. Exendin-4 and GLP-1 stimulate other biological responses with similar efficacy, such as insulin secretion from β-cells in islets [41, 42]. Because its amino acid sequence is somewhat different from GLP-1, it is more resistant

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GLP-1      HAEG-TFTSD-VSSYL- EGQAA-KEFIA-WLVKG-RG
Exendin-4  HGEG-TFTSD-LSKQM-EEEAV-RLFIE-WLKNG-GPSSG-APPPS
```

Figure A-3: Amino acid sequences of GLP-1 and Exendin-4

to enzymatic degradation in the body, leading to an increased half-life of 2.4 hours following subcutaneous injection [113].

Exendin-4 has been found to be useful for type II diabetic patients, because it improves beta-cells' sensitivity to insulin [41] and was recently approved by the FDA for use as a treatment option for Type II diabetic patients to provide glycemic control in patients who are taking metformin, sulfonylurea, or a combination of both [113]. Marketed under the name BYETTA™, it is the first in a class of drugs known as incretin mimetics developed by Amylin Pharmaceuticals, Inc. and Eli Lilly and Company [113].

A.3. Materials and Methods

A.3.1. Materials

A heterobifunctional ω-amino-α-carboxyl poly(ethylene glycol) (PEG; MW 3400; HCl · NH2–PEG3400–COOH) was purchased from Shearwater Polymers (Huntsville, AL). Exendin-4 was purchased from American Peptide Co. (Sunnyvale, CA). Dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), triethylamine (TEA), anhydrous ether, dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), Hank's Balanced Saline Solution without calcium and magnesium (HBSS), Hank's Balanced Saline Solution (HBSS), Sodium Chloride, Collagenase type V, Ficoll type 400-DL, D-Glucose, sodium bicarbonate ($NaCHO_3$), bovine serum albumin, RPMI 1640 medium powder, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), sodium phosphate dibasic (Na_2HPO_4), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (EGTA), potassium phosphate monobasic (KH_2PO_4), magnesium

chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), potassium chloride (KCl), calcium chloride ($CaCl_2$), zinc chloride ($ZnCl_2$), and phosphate buffered saline (PBS) powder were purchased from Sigma-Aldrich Co. (St. Louis, MO). Penicillin/Streptomycin antibiotics and Fetal Bovine Serum were purchased from Gibco-BRL (Grand Island, NY). Spectrapor dialysis tubing (MWCO 15,000) was purchased from Spectrum Laboratories (Rancho Dominguez, CA). Sprague-Dawley Male Rats were purchased from Charles-River Laboratories (Wilmington, MA). Insulin Radioimmunoassay kits were purchased from MP Biomedical (Solon, OH).

A.3.2. Synthesis of Exendin-4/Polymer Conjugate

The poly(N-vinyl-2-pyrrolidone-co-acrylic acid) (VA) polymer backbone was the same material previously created in our lab [114]. The backbone was activated and attached to poly(ethylene glycol) (PEG) using a previously reported method [13]. In brief, 1.5g VA was dissolved in 100mL of DMF with the addition of DCC, NHS, and TEA. After reacting for 48 hours at room temperature under nitrogen atmosphere, the mixture was precipitated twice in anhydrous diethyl ether, dried under vacuum, and stored until further use. To link the PEG_{3400} spacer, 130mg of PEG was dissolved in 10mL DMF with 0.1mL TEA for 1 hour and then mixed with activated VA in DMF with TEA. The reaction was carried out at room temperature for 48 hours under nitrogen atmosphere. The product was dialyzed against distilled water for 5 days (MWCO 15kDa). The dialyzed solution was collected and lyophilized and the PEG-VA (VAP) powder was stored until further use.

The final step was to conjugate Exendin-4 to the PEG-VA construct. Twenty milligrams of VAP was dissolved in 10mL dimethyl sulfoxide (DMSO) containing TEA and reacted for 24 hours at room temperature under nitrogen atmosphere to activate the PEG. Exendin-4 (5mg) was added and the reaction was continued for another 24 hours at room temperature under nitrogen atmosphere. The polymer solution was dialyzed against distilled water for 5 days using MWCO 15,000 membrane. The final product VAPE was obtained following lyophilization and stored at –20°C until further use. Chemical conjugation of the peptide was confirmed by HPLC. Exendin-4 content in the conjugate was determined by UV spectroscopy, and bioactivity of VAPE was confirmed through a glucose-challenge test using rat pancreatic islets.

A.3.3. Islet Isolation

Rat islets were freshly isolated from the pancreas of male Sprague-Dawley rats using standard collagenase digestion and Ficoll gradient methods [115, 116]. Islets were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% Penicillin and Streptomycin for 48 hours prior to experimentation.

A.3.4. Free Islet Glucose Challenge Experiment

Following culture, 20 islets were plated per well in 24-well tissue culture plates and rinsed twice with phosphate-buffered saline to remove any residual medium. Islets were glucose-starved by incubation in Kreb's Ringer HEPES (KRH) buffer containing 2.8mM glucose (50G) for 30 minutes at 37°C. Incubation medium was collected, and islets were placed in 16.8mM (300G) glucose-containing KRH buffer to simulate post-

prandial conditions. The following stimulants were added to the wells: no stimulant, free native Exendin-4, or VAPE in varying concentrations from 1nM to 1µM to mimic typical fasting and post-meal circulating GLP-1 levels. Islets were incubated for 1 hour at 37°C, medium samples were collected and the islets were rinsed and incubated with 50G buffer for 10 minutes before the next buffer was added. The same procedure was repeated using 11.2mM glucose-KRH buffer (200G) and 5.6mM glucose-KRH buffer (100G) and samples were collected after each buffer change.

A.3.5. Islet Encapsulation

Islets were isolated in the same manner described above followed by 48 hours incubation at 37°C prior to encapsulation. Islets were collected and rinsed with 0.9% normal saline solution twice before encapsulation to remove any residual medium. Islets were mixed with 4% alginate solution to make a final alginate concentration of 3%. For control capsules, islets only were mixed with alginate, for capsules containing VAPE - the VAPE was mixed with the islets prior to adding alginate. The islet/alginate mixture was loaded into the Nisco Encapsulation Unit type V1 (Zurich, Switzerland) and capsules were extruded (flowrate - 5.0mL/hr, 60% agitation, 5.6mV) through a 0.5mm OD needle into 50mM $CaCl_2$ solution and stirred for 20 minutes to allow capsule hardening. Capsules were collected and rinsed twice with 0.9% normal saline and incubated in 0.05% Poly-L-Lysine (PLL) solution for 10 minutes. Capsules were rinsed twice with 0.9% normal saline and incubated with 0.3% alginate solution for 10 minutes. The capsules were coated once more with PLL and then alginate to create the five-layer capsule membrane (scheme 1). The final step was to expose the capsules to EGTA for 5 minutes

to liquefy the capsule core. Capsules were collected and placed in RPMI1640 medium and stored in the incubator at 37°C for 24 hours before further experimentation.

A.3.6. Encapsulated Islet Glucose Challenge

Encapsulated islets were placed in 24-well tissue culture plates for all glucose challenge experiments. Each well received 20 encapulated islets and experimental conditions were run at least in triplicate. Cell culture well inserts were used to minimize capsule loss during experimentation. Glucose challenge experiments were carried out in the same manner as described above for free islets. Briefly, capsules were rinsed in 2.8mM glucose KRH medium (50G) to remove any residual medium, and then incubated in fresh 50G medium for 30 minutes to simulate starvation glucose levels. Capsules were then cycled through 16.8mM (300G) glucose, 11.2mM (200G) glucose, and 5.6mM (100G) glucose containing KRH buffer. Capsules were incubated in 50G buffer for 10 minutes in between each buffer change to stop any stimulatory effect from the previous conditions from carrying over. Buffer samples were collected from the initial 50G incubation and after each of the 300G, 200G, and 100G incubations and stored at -20°C until analysis using insulin radioimmunoassay kits. All samples collected from the glucose challenge experiments were assayed for insulin content using I125 labeled insulin antibody radioimmunoassay kits. Samples were read using a Beckman-Coulter Gamma Counter.

The statistical significance of the data was evaluated by conducting unpaired Student's t-test with a confidence level of $p<0.05$.

A.4. Results and Discussion

A.4.1. Synthesis and Characterization of Exendin-4/polymer Conjugate

The previously synthesized poly(N-vinyl-2-pyrrolidone-co-acrylic acid) (VA) polymer backbone was activated and conjugated to polyethylene glycol (MW3400) (PEG) through an amide linkage to form VA-PEG (VAP). Conjugation of the PEG to the backbone was verified by ^{1}H-NMR and mass spectrometry. NMR Spectra were taken in d_6-DMSO and the formation of VAP was confirmed. After determining that the PEG had been attached to the backbone, the carboxyl-end of the conjugate was activated and bound to Exendin-4 peptide through a second amide linkage to form the Exendin-4/VAP conjugate (VAPE), which was verified by ^{1}H-NMR and HPLC. Conjugation of Exendin-4 was confirmed by the NMR spectra taken in d_6-DMSO. HPLC analysis showed that the VAPE conjugate displayed a peak corresponding to Exendin-4 that was absent in samples containing the polymer backbone only (VA) and the backbone conjugated to PEG (VAP), indicating the peptide was bound to the backbone. Exendin-4 contains a tryptophan residue which displays characteristic absorption at 280nm wavelength after exposure to UV. This was useful in determining the amount of Exendin-4 incorporated into the final polymer conjugate, which was determined to be 30% by weight using UV spectroscopy.

Following the formation of the conjugate, peptide activity of Exendin-4 was assessed to determine whether it was still patent or if its bioactivity was lost during conjugation. Free pancreatic islets were subjected to a glucose challenge test to determine insulin output. Islets received either free Exendin-4 peptide or the VAPE conjugate in conjunction with their exposure to buffer containing 16.8mM glucose and the buffer sample was assayed for insulin content using radioimmunoassay techniques. Insulin release was similar for both the free Exendin-4 and VAPE groups ($p=0.92$),

indicating that the peptide retained its bioactivity following the conjugation reaction (figure A-4). Insulin output did not differ significantly between islets exposed to VAPE and control islets following exposure to lower glucose levels (2.8mM and 5.6mM glucose), indicating that as expected, VAPE did not stimulate insulin secretion at lower levels of glucose. However upon increasing glucose levels to 16.8mM, insulin output increased over 300% when islets were exposed to VAPE compared to control islets ($p = 0.0016$) indicating that the VAPE was able to stimulate insulin production in the islets in response to elevated glucose levels (Figure A-4).

A.4.2. Characterization of Encapsulated Islets

Islets were encapsulated in a 5-layer alginate-poly-L-Lysine microcapsule. Following encapsulation islets were incubated for 24 hours before being selected for further experiments. As shown in Figure A-5, capsules were perfectly spherical with 1-2 islets encapsulated within the core. Islets were encapsulated either with or without VAPE and subjected to glucose challenge experiments to see if encapsulation changed the islets' response to increased glucose levels.

Encapsulated islets were also cultured over a few weeks to determine whether the system responded to repeated glucose stimulation over time. Encapsulated islets were subjected to glucose challenge conditions daily over 14 days and insulin secretion was determined by radioimmunoassay. Insulin secretion from the control encapsulated islets decreased rapidly over the 14-day period and continued to decrease over the entire 8 weeks, while insulin secretion from islets encapsulated with VAPE remained relatively constant for the first 14 days and then decreased at a slower rate over the

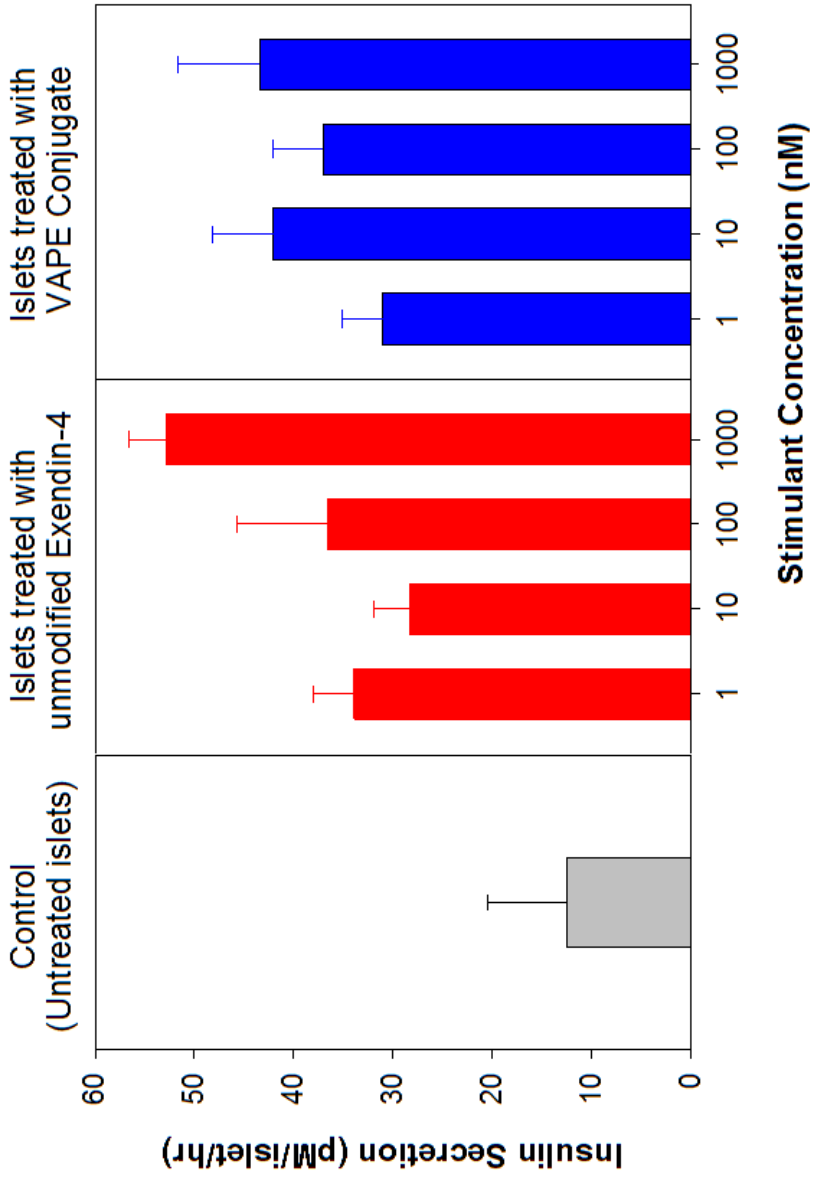

Figure A-4: Insulin secretion following stimulation using unmodified Exendin-4 or Exendin-4/polymer conjugate (VAPE). (n=4, mean ± SD)

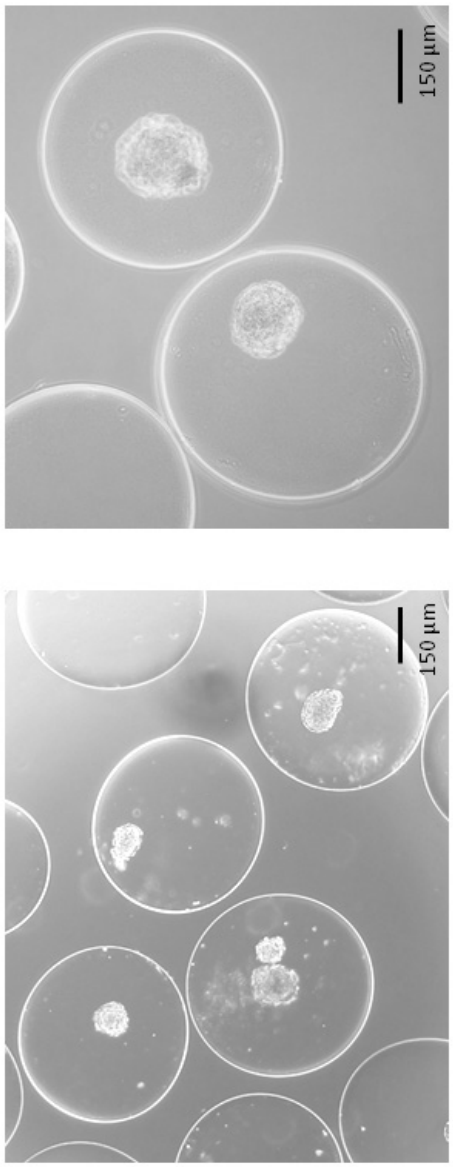

Figure A-5: Encapsulated islets – Control (left) and with VAPE (right)

entire 8 weeks, and remained consistently higher than the control (Figure A-6). In comparing the insulin secretion levels between control encapsulated islets and islets encapsulated with VAPE, the percent of insulin secretion was significantly higher from islets encapsulated with VAPE because insulin secretion remained relatively stable while insulin secretion from the control islets declined sharply after day 5 (Figure A-6). This could be because Exendin-4 had some protective effect on the islet cells and decreased the rate at which beta cells succumbed to apoptosis.

A.5. Conclusions

An Exendin-4/polymer conjugate was successfully created that was effective in significantly increasing the insulin output from pancreatic islets in response to elevated glucose levels. In addition, the conjugate did not have any stimulatory effect on the islets when glucose levels were low, which would be beneficial in preventing accidental hypoglycemic events. Islets encapsulated with VAPE maintained constant insulin secretion levels in response to cyclic changes in glucose concentration over two weeks, indicating continued functionality and responsiveness over time. This polymer may be a good candidate to reduce the number of islets required for a single islet transplantation procedure.

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This work was partially funded by NIH DK 56884. I would also like to gratefully acknowledge Dr. Han Chang Kang for his help with the islet isolation.

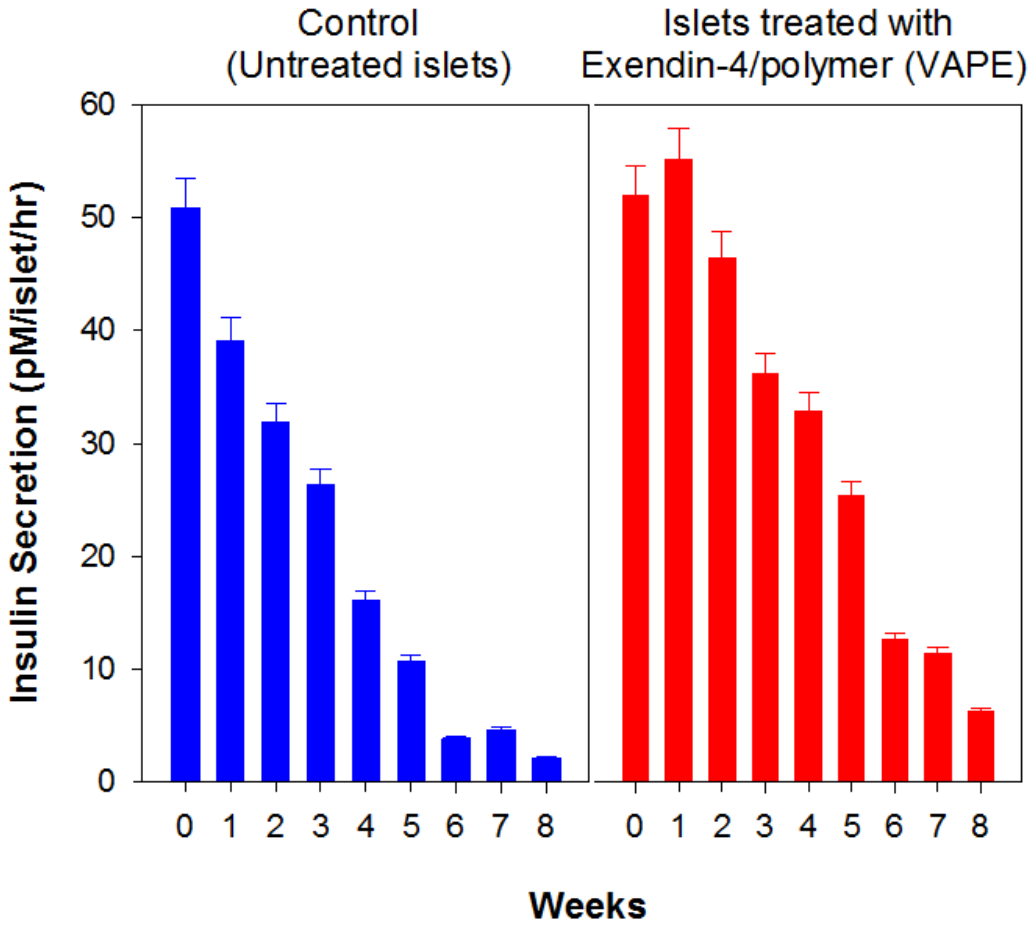

Figure A-6: Insulin secretion from encapsulated islets over 8-week period. (n=4, mean ± SD)

A.7. References

[1] Prevention CfDCa. National Diabetes Fact Sheet: general information and national estimates on diabetes in the United States, 2005. In: Services UDoHaH, editor. Atlanta, GA: Atlanta GA: US Department of Health and Human Services, Centers for Disease Control and Prevention, 2005.

[2] Organization WH. Printable Fact Sheet No. 312, September 2006. http://wwwwhoint/mediacentre/factsheets/fs312/en/, 2006.

[3] Organization WH. Diabetes Action Now Booklet 2004. http://wwwwhoint/diabetes/actionnow/booklet/en/, 2004.

[4] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047-1053.

[5] Figliuzzi M, Cornolti R, Plati T, Rajan N, Adobati F, Remuzzi G, et al. Subcutaneous xenotransplantation of bovine pancreatic islets. Biomaterials 2005;26(28):5640-5647.

[6] Chae SY, Kim SW, Bae YH. Bioactive polymers for biohybrid artificial pancreas. J Drug Target 2001;9(6):473-484.

[7] Wang Z, Zhu T, Rehman KK, Bertera S, Zhang J, Chen C, et al. Widespread and stable pancreatic gene transfer by adeno-associated virus vectors via different routes. Diabetes 2006;55(4):875-884.

[8] Samson SL, Chan L. Gene therapy for diabetes: reinventing the islet. Trends Endocrinol Metab 2006;17(3):92-100.

[9] Isaac JR, Skinner S, Elliot R, Salto-Tellez M, Garkavenko O, Khoo A, et al. Transplantation of neonatal porcine islets and sertoli cells into nonimmunosuppressed nonhuman primates. Transplant Proc 2005;37(1):487-488.

[10] Wang DZ, Skinner S, Elliot R, Escobar L, Salto-Tellez M, Garkavenko O, et al. Xenotransplantation of neonatal porcine islets and Sertoli cells into nonimmunosuppressed streptozotocin-induced diabetic rats. Transplant Proc 2005;37(1):470-471.

[11] Roche E, Santana A, Vicente-Salar N, Reig JA. From stem cells to insulin-producing cells: towards a bioartificial endocrine pancreas. Panminerva Med 2005;47(1):39-51.

[12] Roche E, Jones J, Arribas MI, Leon-Quinto T, Soria B. Role of small bioorganic molecules in stem cell differentiation to insulin-producing cells. Bioorg Med Chem

2006;14(19):6466-6474.

[13] Kim S, Kim SW, Bae YH. Synthesis, bioactivity and specificity of glucagon-like peptide-1 (7-37)/polymer conjugate to isolated rat islets. Biomaterials 2005;26(17):3597-3606.

[14] Dusseault J, Leblond FA, Robitaille R, Jourdan G, Tessier J, Menard M, et al. Microencapsulation of living cells in semi-permeable membranes with covalently cross-linked layers. Biomaterials 2005;26(13):1515-1522.

[15] Zimmermann H, Zimmermann D, Reuss R, Feilen PJ, Manz B, Katsen A, et al. Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation. J Mater Sci Mater Med 2005;16(6):491-501.

[16] Goosen MFA, O'Shea GM, Gharapetian H, Chou S, Sun AM. Optimization of microencapsulation parameters: semipermeable microcapsules as a bioartificial pancreas. Biotech Bioeng 1985;27(2):146-150.

[17] Goosen MFA. In: Williams DF, editor. CRC Critical Reviews in Biocompatibility. Boca Raton, FL: CRC Press, 1987. p. 1-24.

[18] King GA, Daugulis AJ, Faulkner P, Goosen MFA. Alginate-Polylysine microcapsules of controlled membrane molecular weight cutoff for mammalian cell culture engineering. Biotech Progress 1987;3(4):231-240.

[19] Goosen MFA, King GA, McKnight CA, Marcotte N. Animal cell culture engineering using alginate polycation microcapsules of controlled membrane molecular weight cut-off. J Membrane Sci 1989;41:323-343.

[20] Orive G, Tam SK, Pedraz JL, Halle JP. Biocompatibility of alginate-poly-l-lysine microcapsules for cell therapy. Biomaterials 2006;27(20):3691-3700.

[21] de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 2006;27(32):5603-5617.

[22] de Vos P, Hamel AF, Tatarkiewicz K. Considerations for successful transplantation of encapsulated pancreatic islets. Diabetologia 2002;45(2):159-173.

[23] Omer A, Keegan M, Czismadia E, de Vos P, Van Rooijen N, Bonner-Weir S, et al. Macrophage depletion improves survival of porcine neonatal pancreatic clusters contained in alginate macrocapsules transplanted into rats. Xenotransplantation 2003;10(3):240-251.

[24] Toso C, Oberholzer J, Ceausoglu I, Ris F, Rochat B, Rehor A, et al. Intra-portal injection of 400um microcapsules in a large animal model. Transpl Int 2003;16(6):405-410.

[25] Association AD. Type I diabetes conditions and treatments. 2006. http://www.diabetes.org/type-1-diabetes/islet-transplants.jsp

[26] (NIDDK) NIoDaDaKD, NIH. NIH Publication No. 04-4693. 2003.

[27] Ahrén B. Glucagon-like peptide-1 (GLP-1): a gut hormone of potential interest in the treatment of diabetes. Bioessays 1998;20(8):642-651.

[28] Holst JJ. Glucagon-like peptide 1. Curr Opin Endocrinol Diabetes 1997;4:256-261.

[29] Holst JJ. Glucagon-like peptide-1 (GLP-1), a newly discovered GI hormone. Gastroenterology 1994;107:1848-1855.

[30] Kieffer TJ, Habener F. The glucagon-like peptides. Endocr Rev 1999;20:93-102.

[31] Ahrén B, Pacini G. Dose-related effects of GLP-1 on insulin secretion, insulin sensitivity, and glucose effectiveness in mice. Am J Physiol 1999;277(6 Pt 1):E996-E1004.

[32] Del Prato S, Pulizzi N. The place of sulfonylureas in the therapy for type 2 diabetes mellitus. Metabolism 2006;55(5 Suppl 1):S20-27.

[33] Willms A, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 3 (noninsulin-dependent) diabetic patients. J Clin Endocrinol Metab 1996;81:327-332.

[34] Orsakov C. Glucagon-like peptide-1, a new hormone of the enteroinsular axis. Diabetologia 1992;35:701-711.

[35] Nauck MA. Is glucagons-like peptide 1 an incretin hormone? Diabetologia 1999;42:373-379.

[36] Naslund E, Bogefors J, Skogar S, Gryback P, Jacobsson J, Holst JJ, et al. GLP-1 slows solid gastric emptying and inhibits insulin, glucagon, and PYY release in humans. Am J Physiol 1999;277(3 Pt 2):R910-916.

[37] Raufman J-P, Jensen RT, Sutliff VE, Pisano JJ, Gardner JD. Actions of Gila monster venom on dispersed acini from guinea pig pancreas. Am J Physiol 1982;242(5):G470-474.

[38] Hoshino M, Yanaihara C, Hong Y-M, Kishida S, Katsumaru Y, Vandermeers A, et al. Primary structure of helodermin, a VIP-secretin-like peptide isolated from Gila monster venom. . FEBS Lett 1984;178(2):233-239.

[39] Eng J, Kleinman WA, Singh L, Singh G, Raufman J-P. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. J Biol Chem 1992;267(11):7402-7405.

[40] Fehmann HC, Jiang J, Schweinfurth J, Wheeler MB, Boyd AE, Göke B. Stable expression of the rat GLP-1 receptor in CHO cells: activation and binding characteristics utilizing GLP-1(7-36)-amide, oxyntomodulin, exendin-4, and exendin(9-39). Peptides 1994;15(3):453-456.

[41] Göke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, et al. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-37)-amide receptor of insulin-secreting beta-cells. J Biol Chem 1993;268(26):19650-19655.

[42] Schepp W, Schmidtler J, Riedel T, Dehne K, Schusdziarra V, Holst JJ, et al. Exendin-4 and exendin-(9-39)NH2: agonist and antagonist, respectively, at the rat parietal cell receptor for glucagon-like peptide-1-(7-36)NH2. Eur J Pharmacol 1994;269(2):183-191.

[43] Kroker M. Overview of current therapy in diabetes mellitus. EDTNA ERCA J 2004;30(3):124-127.

[44] Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54(Suppl 2):S97-107.

[45] Nathan DM. Long-term complications of diabetes mellitus. New Engl J Med 1993;328(23):1676-1685.

[46] Barrett-Connor E, Orchard T. Insulin-dependent diabetes mellitus and ischemic heart disease. Diabetes Care 1985;8:65-70.

[47] Barrett-Connor E, K.T. K. Diabetes mellitus: an independent risk factor for stroke? Am J Epidemiol 1988;128(1):116-123.

[48] Bild DE, Selby JV, Sinnock P, Browner WS, Braveman P, Showstack JA. Lower extremity amputation in people with diabetes. Epidemiology and prevention. Diabetes Care 1989;12(1):24-31.

[49] Geiss LS, Herman WH, Teutsch SM. Diabetes and renal mortality in the United States. Am J Public Health 1985;75(11):1325-1326.

[50] Moss SE, Klein R, Lein BE. The incidence of vision loss in a diabetic population. Ophthalmology 1988;95(10):1340-1348.

[51] Sutherland DE, Gruessner RW, Gruessner AC. Pancreas transplantation for treatment of diabetes mellitus. World J Surg 2001;25(4):487-496.

[52] Foundation NK. 2006. http://www.kidney.org/atoz/atozItem.cfm?id=162

[53] Allen RM. Pancreas Transplantation. In: Forsythe JLR, editor. Transplantation Surgery. London: W. B. Saunders, 1997. p. 167-201.

[54] Gaber AO, Wicks MN, Stratta RJ, Gaber LW, Shokouh-Amiri MH, Hathaway DK, et al. Pancreas-kidney transplantation in diabetic uremic patients: An overview. In: Malluche HH, Sawaya RM, Sayegh MH, Ismail N, editors. Clinical Nephrology, Dialysis and Transplantation. Landshut, Germany: Dustri-Verlag, 1999. p. 1-26.

[55] Gaber AO, Shokouh-Amiri MH, Hathaway DK, Hammontree L, Kitabchi AE, Gaber LW, et al. Results of pancreas transplantation with portal venous and enteric drainage. Ann Surg 1995;221(6):622-624.

[56] Robertson RP, Davis C, Larsen J, Stratta R, Sutherland DE. Pancreas and islet transplantation for patients with diabetes. Diabetes Care 2000;23(1):112-116.

[57] Guilhem I, Leguerrier AM, Lecordier F, Poirier JY, Maugendre D. Technical risks with subcutaneous insulin infusion. Diabetes Metab 2006;32(3):279-284.

[58] Vasudevan AR, Burns A, Fonseca VA. The effectiveness of intensive glycemic control for the prevention of vascular complications in diabetes mellitus. Treat Endocrinol 2006;5(5):273-286.

[59] Stitt AW, Curtis TM. Advanced glycation and retinal pathology during diabetes. Pharmacol Rep 2005;57 Suppl:156-168.

[60] Pfeiffer EF. Artificial pancreas, glucose sensors, and the impact upon diabetology. Int J Artif Organs 1993;16(9):636-644.

[61] Pfeiffer EF, Kerner W, Beischer W. Substitution of islet cell function by mechanical device: extracorporeal artificial pancreas. Adv Exp Med Biol 1979;119:501-508.

[62] Kruse-Jarres JD, Braun G, Naegele R, Bresch M, Lehmann U. Blood glucose monitoring and computer regulation by means of an artificial endocrine pancreas. Horm Metab Res Suppl 1979(8):42-45.

[63] Kerner W, Beischer W, Herfarth C, Pfeiffer EF. Application of an artificial endocrine pancreas in the management of the diabetic surgical patient. Horm Metab Res Suppl 1979(8):159-161.

[64] Abel PU, von Woedtke T. Biosensors for in vivo glucose measurement: can we cross the experimental stage? Biosens Bioelectron 2002;17(11-12):1059-1070.

[65] Hunkeler D. Allo transplants xeno: as bioartificial organs move to the clinic. Introduction. . Ann N Y Acad Sci 2001;944:1-6.

[66] Kizilel S, Garfinkel M, Opara E. The bioartificial pancreas: progress and challenges. Diabetes Technol Ther 2005;7(6):968-985.

[67] Qian J, Hashimoto T, Fujiwara H, Hamaoka T. Studies on the induction of tolerance of alloantigens. I. The abrogation of potentials for delayed-type-hypersensitivity response to alloantigens by portal venous inoculation with allogeneic cells. J Immunol 1985;134(6):3656-3661.

[68] De Vos P, Van Straaten JF, Nieuwenhuizen AG, de Groot M, Ploeg RJ, De Haan BJ, et al. Why do microencapsulated islet grafts fail in the presence of fibrotic overgrowth? Diabetes 1999;48(7):1381-1388.

[69] Lanza RP, Ecker DM, Kuhtreiber WM, Marsh JP, Ringeling J, Chick WL. Transplantation of islets using microencapsulation: studies in diabetic rodents and dogs. J Mol Med 1999;77(1):206-210.

[70] Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210(4472):908-910.

[71] Lanza RP, Sullivan SJ, Chick WL. Perspectives in diabetes: islet transplantation with immunoisolation. Diabetes 1992;41(12):1503-1510.

[72] O'Shea GM, Sun AM. Encapsulation of rat islets of Langerhans prolongs xenograft survival in diabetic mice. Diabetes 1986;35(8):943-946.

[73] Dufrane D, van Steenberghe M, Goebbels R, Saliez A, Guiot Y, Gianello P. The influence of implantation site on the biocompatibility and survival of alginate encapsulated pig islets in rats. Biomaterials 2006;27(17):3201-3208.

[74] Leicester M. Infection & immunity: humoral immunity. 2004. http://www-micro.msb.le.ac.uk/MBChB/3b.htm

[75] Desai TA, Ferrari M, Cheung K, Chu WH. In vitro immunoisolative properties of microfabricated biocapsules. 19th International Conference - IEEE/EMBS, 1997.

[76] Lanza RP, Kuhtreiber WM, Ecker DM, Staruk JE, Chick WL. Xenotransplantation of porcine and bovine islets without immunosuppression using uncoated alginate microspheres. Transplantation 1995;59(10):1377-1384.

[77] Ryan EA, Lakey JRT, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 2002;51(7):2148-2157.

[78] De Vos P, Van Schilfgaarde R. Biocompatibility issues. In: Chick WL, editor. Cell Encapsulation Technology and Therapeutics. Boston: Birkhauser, 1999. p. 63-79.

[79] Sun AM, O'Shea GM, Goosen MF. Injectable microencapsulated islet cells as bioartificial pancreas. Appl Biochem Biotechnol 1984;10:87-99.

[80] Weber CJ, Norton JE, Reemtsma K, inventors. Method for microencapsulation of cells of tissue. USA, 1993 July 13, 1993.

[81] Sandler S, Andersson A, Eizirik DL, Hellerstrom C, Espevik T, Kulseng B, et al. Assessment of insulin secretion in vitro from microencapsulated fetal porcine islet like cell clusters and rat, mouse, and human pancreatic islets. Transplantation 1997;63(12):1712-1718.

[82] Halle JP, Leblond FA, Pariseau JF, Jutras P, Brabant MJ, Lepage Y. Studies on small (<300 microns) microcapsules: II-Parameters governing the production of alginate beads by high voltage electrostatic pulses. Cell Transplant 1994;3:365-372.

[83] Duvivier-Kali VF, Omer A, Parent RJ, O'Neil JJ, Weir GC. Complete protection of islets against allorejection and autoimmunity by a simple barium alginate membrane. Diabetes 2001;50(8):1698-1705.

[84] Klock G, Frank H, Houben R, Zekorn T, Horcher A, Siebers U, et al. Production of purified alginates suitable for use in immunoisolated transplantation. Appl Microbiol Biotechnol 1994;40(5):638-643.

[85] Kulseng B, Thu T, Espevik T, Skjak-Braek G. Alginate poly-lysine microcapsules as immune barrier: permeability of cytokines and immunoglobins over the capsule membrane. Cell Transplant 1997;6(4):387-394.

[86] Sawhney AS, Hubbell JA. Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompatibilitiy of poly(L-lysine)-alginate microcapsule membranes. Biomaterials 1992;13(12):863-870.

[87] Govan JRW, Fyfe JAM, Jarman TR. Isolation of alginate-producing mutants of *Pseudomonas fluorescens, pseudomonas putida,* and *Pseudomonas mendonica.* J Gen Microbiol 1981;125(1):217-220.

[88] Smidsrod O, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8(3):71-78.

[89] Thu B, Bruheim P, Espevik T, Smidsrod O, Soon-Shiong P, Skjak-Braek G. Alginate polycation microcapsules. I. Interaction between alginate and polycation. Biomaterials 1996;17(10):1031-1040.

[90] Martinsen A, Skjak-Braek G, Smidsrod O. Alginate as Immobilization material: I correlation between chemical and physical properties of alginate beads. Biotech Bioeng 1989;33:79-83.

[91] Thu B, Bruheim P, Espevik T, Smidsrod O, Soon-Shiong P, Skjak-Braek G. Alginate polycation microcapsules II: Some functional properties. Biomaterials 1996;17(10):1069-1079.

[92] Vandenbossche GM, Bracke ME, Cuvelier CA, Bortier HE, Mareel MM, Remon JP. Host reaction against empty alginate-polylysine microcapsules. Influence of preparation procedure. J Pharm Pharmacol 1993;45:115-120.

[93] Halle JP, Leblond FA, Landry D, Fournier A, Chevalier S. Studies of 300-um microcapsules: I. use of arginine esterase release by microencapsulated prostatic cells as a measure of membrane permeability. Transplant Proc 1992;24:2930-2932.

[94] Strand BL, Ryan TL, In't Veld P, Kulseng B, Rokstad AM, Skjak-Braek G, et al. Poly-L-Lysine induces fibrosis on alginate microcapsules via the induction of cytokines. . Cell Transplant 2001;10(3):263-275.

[95] Rhodes CJ, White MF. Molecular insights into insulin action and secretion. Eur J Clin Invest 2002;32(S3):3-13.

[96] Perfetti R, Merkel P. Glucagon-like peptide-1: a major regulator of pancreatic β-cell function. Eur J Endocrinol 2000;143(6):717-725.

[97] Nauck MA, Kleine N, Orskov C, Holst JJ, Willms B, Creutzfeldt W. Normalization of fasting hyperglycemia by exogenous glucagons-like peptide-1 [7-36 amide] in type 2 (non-insulin dependent) diabetic patients. Diabetologia 1993;36:741-744.

[98] Fehmann HC, Goke R, Goke B. Cell and molecular biology of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulin releasing polypeptide. Endocr Rev 1995;16:390-410.

[99] Widmann C, Dolci W, Thorens B. Desensitization and phosphorylation of the glucagon-like peptide-1 (GLP-1) receptor by GLP-1 and 4-phorbol 12-mystriate 13-acetate. Mol Endocrinol 1996;10(1):62-75.

[100] Thorens B. Expression cloning of the pancreatic beta-cell receptor for the gluco-incretin hormone glucagon-like peptide 1. PNAS 1992;89:8641-8645.

[101] Holz GG, Leech CA, Habener JF. Activation of a cAMP-regulated Ca2+ -signaling pathway in pancreatic beta-cells by the insulinotropic hormone glucagons-like peptide-1. J Biol Chem 1995;270:17749-17757.

[102] Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W. Preserved incretin activity of glucagon-like peptide-1 [7-36 amide] but not synthetic human gastric inhibitory polypeptide in patients with type 2 diabetes mellitus. J Clin Invest 1993;91(1):301-307.

[103] Wettergren A, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ. Truncated GLP-1 (proglucagon 78-107 amide) inhibits gastric and pancreatic function in man. Dig Dis Sci 1993;38(4):665-673.

[104] Nauck MA. Therapeutic potential of glucagons-like peptide 1 in type 2 diabetes. Diabetic Med 1996;13:S39-43.

[105] Nauck MA, Heimesaat MM, Behle K, Holst JJ, Nauck MS, Ritzel R, et al. Effects of glucagon-like peptide 1 on counterregulatory hormone responses, cognitive functions, and insulin secretion during hyperinsulinemic, stepped hypoglycemic clamp experiments in healthy volunteers. J Clin Endocrinol Metab 2002;87(3):1239-1246.

[106] Drucker DJ. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. Mol Endocrinol 2003;17:161-171.

[107] Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, et al. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 1996;379(6560):69-72.

[108] Flint A, Raben A, Astrup A, Holst JJ. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest 1998;101(3):515-520.

[109] Drucker DJ. Glucagon-like peptide-1 and the islet beta-cell: augmentation of cell proliferation and inhibition of apoptosis. Endocrinology 2003;144:5145-5148.

[110] Tourrel C, Bailbe D, Meile MJ, Kergoat M, Portha B. Glucagon-like peptide-1 and exendin-4 stimulate beta-cell neogenesis in streptozotocin-treated newborn rats resulting in persistently improved glucose homeostasis at adult age. Diabetes 2001;50(7):1562-1570.

[111] Tourrel C, Bailbe D, Lacorne M, Meile MJ, Kergoat M, Portha B. Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the beta-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. Diabetes 2002;51(5):1443-1452.

[112] De Leon DD, Deng S, Madani R, Ahima RS, Drucker DJ, Stoffers DA. Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. Diabetes 2003;52(2):365-371.

[113] Egan JM, Meneilly GS, Elahi D. Effects of 1-mo bolus subcutaneous administration of exendin-4 in type 2 diabetes. Am J Physiol Endocrinol Metab 2003;284(6):E1072-1079.

[114] Park KH, Kim SW, Bae YH. Interaction of sulfonylurea-conjugated polymer with insulinoma cell line of MIN6 and its effect on insulin secretion. J Biomed Mater Res 2001;55(1):72-78.

[115] Gotoh M, Maki T, Satomi S, Porter J, Bonner-Weir S, O'Hara CJ, et al. Reproducible high yield of rat islets by stationary in vitro digestion following pancreatic ductal or portal venous collagenase injection. Transplantation 1987;43(5):725-730.

[116] Shibata A, Ludvigsen Jr. CW, Naber SP, McDaniel ML, Lacy PE. Standardization fo a digestion-filtration method for isolation of pancreatic islets. Diabetes, 1976. p. 667-672.